

**First evidence of an altered microbiota and intestinal damage and its link to absence epilepsy
in a genetic animal model, the WAG/Rij rat**

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Abstract

Objective: A large number of studies have highlighted the important role of the gut microbiota in the pathophysiology of neurological disorders, suggesting that its manipulation might serve as a treatment strategy. We hypothesized that the gut microbiota participates in absence seizure development and maintenance in the WAG/Rij rat model and tested this hypothesis by evaluating potential gut microbiota and intestinal alterations in the model, as well as measuring the impact of microbiota manipulation using fecal microbiota transplantation (FMT).

Methods: Initially, gut microbiota composition and intestinal histology of WAG/Rij rats (a well-recognized genetic model of absence epilepsy) were studied at 1-, 4- and 8-months of age in comparison to non-epileptic Wistar rats. Subsequently, in a second set of experiments, at 6-months of age, untreated Wistar or WAG/Rij rats treated with ethosuximide were used as gut-microbiota donors for FMT in WAG/Rij rats and electroencephalographic recordings were obtained over 4 weeks. At the end of FMT, stool and gut samples were collected, absence seizures were measured on EEG recordings and microbiota analysis and histopathological examinations were performed.

Results: Gut microbiota analysis showed differences in β -diversity and specific phylotypes at all ages considered and significant variances in the Bacteroidetes/Firmicutes ratio between Wistar and WAG/Rij rats. FMT, from both Wistar and ETH-treated WAG/Rij donors to WAG/Rij rats, significantly decreased the number and duration of seizures. Histological results indicated that WAG/Rij rats were characterized by intestinal villi disruption and inflammatory infiltrates already at 1-month of age before seizure occurrence; FMT partially restored intestinal morphology while also significantly modifying gut microbiota and concomitantly reducing absence seizures.

Significance: Our results demonstrate for the first time, that the gut microbiota is modified and contributes to seizure occurrence in a genetic animal model of absence epilepsy and that its manipulation may be a suitable therapeutic target for absence seizure management.

Keywords: Gut microbiota; Microbiota-gut-brain axis; Fecal microbiota transplantation (FMT); Seizures; inflammation.

Introduction

Epilepsy is one of the most common neurological diseases, characterized by predisposition to generate epileptic seizures¹. Several causative mechanisms have so far been discovered such as neural network alteration, neuroinflammation, an altered release of neurotransmitters and cell death; however, many others are under investigation and undoubtedly further studies are warranted to define new mechanisms and therefore point out new potential therapeutic targets².

Inflammatory processes within the brain constitute a common and crucial mechanism in the pathophysiology of seizures and epilepsy development and also peripheral inflammation appears to contribute to brain hyperexcitability^{3,4}. An example is represented by the increased neuronal hyperexcitability found in animal models of intestinal inflammation^{5,6}, which is further confirmed by the increased incidence of epilepsy in patients suffering from inflammatory bowel diseases⁷. It was previously suggested that chronic intestinal inflammation migrates to other organs, including the brain, and decreases the epileptogenic threshold^{4,6}; these studies have demonstrated that increased seizure susceptibility and neuronal excitability were significantly correlated with the severity of the peripheral inflammation and were reversed with the natural resolution of gut inflammation^{6,8}.

More recently, there has been a growing body of evidence linking the gut microbiota, a complex group of symbiotic microorganisms colonizing the gastrointestinal tract, and brain diseases including epilepsy^{9,10} with a wider definition of the gut microbiota-brain (GMB) axis and its understanding. Notwithstanding, intestinal inflammation as well as other bowel diseases and syndromes are linked to an alteration in the gut microbiota composition¹¹.

The microbiota regulates the central nervous system (CNS) through metabolites, neuroactive molecules and inflammatory factors. The CNS, in turn, regulates the gut microbiota through the vagus nerve and hypothalamic–pituitary–adrenal axis (HPA axis). Epidemiological, clinical and experimental animal studies have revealed the existence of communication pathways between gut and brain that depend on signals from neural, hormonal and immune systems and from the microbiota itself.

Over the last decade, a growing number of studies have found that the gut microbiota is closely connected with brain health, and alteration of gut bacteria composition has been associated with different neurological diseases including neurodevelopmental disorders (*e.g.* autism spectrum disorder)¹² neuropsychiatric¹³ and neurodegenerative conditions (*e.g.* Parkinson's disease; Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis)¹⁴. Restoring a healthy microbial community is therefore a promising therapeutic strategy for diseases related to gut alteration. However, little is known about the role of gut bacteria in epilepsy⁹. Recently, an altered composition of the gut microbiome in patients with drug-resistant epilepsy has been demonstrated; it was characterized by a significantly increased abundance of numerous rare bacteria (Firmicutes) and decreased normal commensal bacteria suggesting that *dysbiosis* could be involved in drug-resistant epilepsy¹⁵.

WAG/Rij rats represent a well validated genetic animal model of absence epilepsy, genetically determined epileptogenesis and neurological/psychiatric comorbidities^{16,17}. In this strain, absence seizures spontaneously appear around the age of 2-3 months in all rats and increase over time up to the age of 6 months reaching a plateau, although absence seizures have a slower increase with aging. It has been established that rats of this strain undergo a genetically determined epileptogenic process which is very likely occurring at all ages with different characteristics and can be influenced by pharmacological treatments but also environmental interventions suggesting a potential epigenetic influence^{16,17}. Furthermore, we previously demonstrated that alpha-lactalbumin, a whey protein rich in tryptophan, was able to prevent epileptogenesis in this model and the same protein is known to modify gut microbiota¹⁸⁻²⁰ while its anticonvulsant activity was linked to a reduction in intestinal inflammation⁵.

We hypothesized that gut microbiota participates in absence seizures development and maintenance in the WAG/Rij rat model and tested this hypothesis by evaluating potential gut microbiota and intestinal alterations in the model as well as measuring the impact of microbiota manipulation using fecal microbiota transplantation (FMT).

Materials and methods

Animals

Male WAG/Rij and Wistar rats (1, 4, 6 and 8 months old; $n= 98$) were obtained from our breeding colony at the University of Catanzaro from rat progenitors originally purchased from Charles River Laboratories S.r.l. (Calco, Lecco, Italia), breeding conditions and authorization are reported in the supporting information. Procedures involving animals and their care were conducted in conformity with the international and national laws and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept).

Experiment#1: Identification of intestinal and gut microbiota alterations in WAG/Rij rats before and after absence seizures onset

In order to determine whether WAG/Rij rats have an altered microbiota contributing to absence epilepsy, we, initially, analyzed fecal microbiota composition, fecal short chain fatty acid (SCFA) levels and the gut morphology/histology of colon and ileum tissues, as well as intestinal motility of WAG/Rij rats before (1 month of age) and after absence seizures onset (4 and 8 months of age) in comparison with Wistar rats (non-epileptic rats). Rats were divided into 6 groups of 6 animals each according to the 3 ages and the 2 strains used, while separate groups of rats were used for intestinal motility ($n=4$ for each group).

Experiment#2: gut microbiota manipulation effects on absence seizures in WAG/Rij rats

Considering the microbiota alteration and potential contribution to absence epilepsy observed in *experiment#1*, in order to further define this link, we analyzed whether exogenous bacteria could reshape the gut microbial composition of epileptic rats, by transplanting the fecal microbiota from Wistar non-epileptic rats to recipient WAG/Rij rats and measuring its impact on absence seizures. Furthermore, we studied the effects of microbiota transplantation from WAG/Rij ethosuximide

(ETH)-treated rats to recipient WAG/Rij rats. We used a total of 50 rats of 6 months of age divided as follows:

1) WAG/Rij rats:

- CTRL group for EEG ($n=5$)
- Fecal donors for Wistar recipient rats (used at 8 months in experiment#1; $n=6$)
- Antibiotic treated group ($n=5$)
- Antibiotic treatment and FMT from Wistar rats ($n=5$)
- WAG/Rij rats treated with ETH as fecal donors ($n=6$)
- ETH-treated fecal donors for WAG/Rij recipient rats ($n=6$)

2) Wistar rats:

- Fecal donors for WAG/Rij recipient rats (used at 8 months in experiment#1; $n=6$)
- Antibiotics-treated group ($n=5$)
- Antibiotic treatment and FMT from WAG/Rij rats ($n=6$)

Antibiotics treatment was conducted for 12 days, and the transplant procedure was performed starting on day 13th, one day after the end of antibiotics treatment, three times a week for 4 consecutive weeks. Fecal samples, for bacterial counts and 16S rDNA sequencing, were collected from all recipient and control rats at each time-point (see Figure S1); *i.e.*, prior to antibiotic administration; immediately after the full antibiotic regimen and before the beginning of FMT; and at 4 weeks post-FMT.

EEG recordings were performed at different times, before the antibiotic treatment (PRE-ABX), after the end of antibiotic treatment (POST-ABX) and every week during the fecal transplantation period for a total of 4 EEG recordings during the transplant procedure (Figure S1). Methodological details on: fecal sample collection, antibiotics treatment, EEG recordings and gut microbiota analysis are reported in the supporting information file available on line.

Intestinal motility and Fecal SCFAs analysis

Upper gastrointestinal transit was measured in WAG/Rij and Wistar rats at 1, 4 and 8 months of age ($n=4$ for each strain and age; for more details see supporting information). Transit was determined by identifying the leading front of an intragastrically administered charcoal meal marker in the small intestine, as previously described²¹. Fecal samples ($n=6$ per group) were obtained at all different time-points, propionate and butyrate concentrations were determined as previously described⁵ (for more details see supporting information).

Histological Analysis and Immunohistochemistry

The ileum 5 cm above the cecum and 5 cm of proximal colon from all established groups were obtained and tissue sections were evaluated by standard light microscopy using a LEICA 6000 microscope (Leica, Germany). Intestinal damage was quantified according to Erben et al.²². Sections of 5-7 μm were de-paraffinized, dehydrated, and submitted to antigen retrieval. Gut morphology and integrity were detected using polyclonal rabbit anti-ZO-1 antibody (Proteintech Abcam, UK). The intensity of staining in slide photographs was analyzed using a computer imaging program (ImageJ_1.41; <http://rsb.info.nih.gov/ij/>).

Microbiota transplantation

Untreated rats from each strain (WAG/Rij and Wistar) and WAG/Rij rats treated for at least 30 days with ETH, were used as intestinal microbiota donors. The animals in the ETH group were treated with ETH at a dose of 300 mg/Kg/day orally²³; this treatment is known to mostly suppress all seizures in adult WAG/Rij rats²³. For FMT of fresh fecal material, donor rats were placed in empty autoclaved cages (no bedding) and allowed to defecate normally.

Fecal samples were freshly prepared on the day of transplant and administered within 3 h. Fecal supernatant used for FMT was given via single oral gavage (1 ml/rat/os), three times per week over

4 weeks²⁴. Rats treated with antibiotic, that did not receive FMT, received oral gavages of vehicle (PBS; for further details see Supporting information). ETH content in the feces of ETH-treated fecal donors for WAG/Rij recipient rats and in the blood of WAG/Rij rats treated with ETH as fecal donors was performed by HPLC analysis by EUREKA s.r.l Lab Division method (for more details see supporting information).

Microbiota sequencing and data analysis

V3-V4 16S rDNA sequences from WAG/Rij and WISTAR fecal samples were obtained and processed as previously described in Coretti et al., 2017²⁵. To avoid sample size biases, a sequence rarefaction procedure was applied using a maximum depth of 10,000 sequences/sample. Alpha diversity metrics, as measures of species heterogeneity in each sample, were assessed on rarefied OTU table using Good's coverage, Observed species and Shannon's diversity index and compared by a two-sample permutation t-test, using 999 Monte Carlo permutations. OTUs diversity among sample communities (beta diversity) was assessed by computing unweight and weighted Unifrac distances and tested using ANOSIM method with 999 permutations. Statistical differences in OTUs frequencies among groups across different taxonomic levels were assessed through one-way ANOVA followed by Tukey's multiple comparison post-hoc tests. SPecies Identification of metaGenOmic amplicons program (SPINGO) (version 1.3) with default parameters was performed to increase taxonomic resolution at species level on a representative sequence of each OTU of key genera (ANOVA p-value < 0.05 and relative abundance > 0.5%) discriminating the groups.

Statistical Analysis

All statistical procedures were performed using GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA, USA). EEG recordings were subdivided into 30 min epochs, and the duration and number of spike wave discharges (SWDs) were evaluated separately for every epoch. Such values

were averaged and data obtained were expressed as mean \pm S.E.M. for every group and compared by 1-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test.

Intestinal transit and SCFA results are expressed as the mean \pm S.E.M. of n experimental replicates.

The significance of differences between groups was determined by two-way ANOVA followed by a Bonferroni's post hoc test for multiple comparisons. The level of statistical significance was $p < 0.05$.

Results

Identification of gut microbiota and intestinal alterations in WAG/Rij rats

We analyzed the fecal microbiota of WAG/Rij rats at baseline (1 month of age) and during development (adult rats, 4 and 8 months of age) in comparison with age-matched Wistar rats. The results showed that for alpha diversity analyses, species richness (number of operational taxonomic units [OTUs]) and degree of evenness (Shannon index) (Table S1) revealed no significant differences between groups, while variances in phylogenetic assortment (beta diversity) in WAG/Rij rats during development were observed (Figure S2). Phylogenetic distances of bacterial species among groups were measured by unweighted and weighted UniFrac analysis and reported in Principal Coordinate Analysis (PCoA) plots (Figure S2A and B, respectively). The ANOSIM R statistics for unweighted UniFrac analysis revealed that both WAG/Rij rats and controls showed a robust and long-lasting reshape of microbiota structure during development (Figure S2A). Notably, as of 1 month of age, gut bacterial assortment of WAG/Rij rats was clearly distinguishable from age-matched controls ($R = 0.525$, $p = 0.008$) and a strong increase in phylogenetic distance of gut microbes was observed in 4 and 8 months old WAG/Rij rats when compared to control rats ($R=0.99$, $p=0.002$ and $R=0.97$, $p=0.004$, respectively; Figure S2A, ANOSIM between genotypes within same age). The main results were corroborated by weighted UniFrac analysis that also takes into account microbial species evenness among groups (Figure S2B). Moreover, weighted beta-diversity analysis of WAG/Rij rats displayed a former (4 months of age) achievement of microbial balance in terms of microbial species evenness with respect to control rats.

Alteration in Bacteroidetes and Firmicutes abundance typifies the WAG/Rij rat microbiota profile

Comparison of gut microbiota composition in the different groups was carried out at phylum level (Figure 1). The distribution of most abundant OTUs for each sample is shown in the heatmap (Figure 1A). Bacteroidetes and Firmicutes were among the most dominant phyla both in WAG/Rij and control rats at 1 month of age (Figure 1B). However, the Bacteroidetes/Firmicutes ratio was significantly lower in juvenile WAG/Rij rats compared to controls, depicting early and definite traits of WAG/Rij rat gut microbiota composition (Figure 1C). Remodeling of gut microbiota profiles in adult WAG/Rij rats, resulting in reduction of Bacteroidetes/Firmicutes ratio, was attributable to a marked decrease of the relative abundance of Bacteroidetes at 4-months of age (47.39% vs 24.83% at 1 and 4 months of age, respectively; $p=0.004$) enduring until 8-months of age (47.39% vs 26.15% at 1 and 8 months of age, respectively; $p=0.005$), and a significant long-lasting increase of Firmicutes (38.9% vs 68.93% at 1 and 4 months of age, respectively with $p=0.0003$; 38.9% vs 64.06% at 1 and 8 months of age, respectively with $p=0.001$; Figure 1B,C); variations in microbiota assortment during adulthood were also observed in control rats even though no significant changes in Bacteroidetes levels were identified (52.01% vs 39.21% at 1 and 4 months of age, respectively with $p=0.187$; 52.01% vs 41.06% at 1 and 8 months of age, respectively with $p=0.335$; Figure 1B, C).

Bacterial phylotypes marking the WAG/Rij rat microbiome

All bacterial genera significantly differing in abundance among WAG/Rij and control rats at the different age points are reported in Table 1. The main differences between the two groups were recognized at 4 months of age where a lower abundance of *Unclassified genus (U.g.) of S24-7* along with higher abundance of *Odoribacter* and *U.g. of Rikenellaceae* within Bacteroidetes phylum were identified in WAG/Rij rats. Notably, re-assortment of bacterial genera within Firmicutes phylum at this developmental stage clearly marked differences in microbiota profiles between WAG/Rij and

control rats, being *U.g.* of *Clostridiales*, *Clostridiaceae* and *Lachnospiraceae* more abundant and *Lactobacillus* and *Phascolarctobacterium* prominently less abundant in WAG/Rij rats. SPINGO classification of significant bacterial species belonging to key genera discriminating WAG/Rij and control rats at all time points is reported in Figure S3.

Intestinal motility and fecal propionate and butyrate concentration

The distance traveled by the charcoal marker in the small intestine 20 min after oral administration was different between Wistar and WAG/Rij rats at 1, 4 and 8 months of age (Figure S4). Intestinal motility was faster in WAG/Rij rats older than 4 months compared with Wistar rats. Regarding propionate and butyrate SCFA concentrations, both were found to be significantly lower in WAG/Rij rats than Wistar rats at all ages considered (Figure S5); furthermore, both were found to significantly decrease with aging in WAG/Rij rats while this was true only for propionate in Wistar rats.

Intestinal histomorphology and zonulin staining in Wistar and WAG/Rij rats at different ages

Histological changes were consistently observed in terminal ileum tissue of WAG/Rij rats in comparison to Wistar rats at 1, 4 and 8 months of age (Figure S6), in all samples analyzed. Images from Wistar rats show an intact ileum epithelium and finger-like villi (Score 0) at all ages (Figure S6A, B and I). In contrast, WAG/Rij rats presented a marked mucosal, submucosal and transmural inflammatory cell infiltration and lymphoid aggregates accompanied by villous broadening (Score about 4; Figure S6C, D and I), with no differences among ages. Moreover, the morphological structure of proximal colon tissues both at 1 and 8 months of age had an intact epithelium and normal morphology of villi for both Wistar and WAG/Rij rats (Figure S7). Of note, in some cases, cell infiltration and mucosal damage could be observed; however, this was not constantly observed and reliable (Figure S7 C). Zonulin staining was substantially in line with the morphological damage observed (Figure S6H and L; Figure S8).

Histomorphology of terminal ileum of Wistar and WAG/Rij rats after FMT

FMT had a substantial impact on ileum morphology; remarkably, WAG/Rij rats receiving feces from both Wistar donors or ETH-treated donors had a significant improvement in the structure of their intestinal tissue with the score being reduced in a similar manner between the two groups. A partial normalization and reduction of inflammatory cell infiltration and lymphoid aggregates was observed displaying a mild alteration of ileal mucosa with normal crypts and moderate villous blunting (score about 2 vs 4 in control rats; Figure S6F, G and I). On the other hand, FMT in Wistar rats from WAG/Rij donors displayed damaged villi with blunting and marked alteration of the architecture and cellular infiltration of mucosa (score 3.4; Figure S6E and I).

Experiment#2: gut microbiota manipulation effects on absence seizures in WAG/Rij rats

Effects of FMT on absence seizures in WAG/Rij rats

EEG recordings performed before any treatment on all WAG/Rij rats (6 months of age and before any treatment or manipulation) showed a mean number of SWDs (nSWDs) of 15.06 ± 1.25 , with a mean total duration (dSWDs) of 102.84 ± 2.01 s and a mean single duration (sSWD) of 6.80 ± 1.24 for a 30-min epoch (Figure 2). Antibiotic mix treatment (12 days) did not have any significant effect on SWDs' incidence and duration both in comparison to aged-matched control group and SWDs parameters of the same animals before antibiotic treatment.

During the 4 weeks of the FMT protocol, control WAG/Rij rats did not show any significant change in the number and the duration of SWDs with a mean number of SWDs (nSWDs) of 14.59 ± 1.72 with a mean total duration (dSWDs) of 112.71 ± 2.23 s and a mean single duration (sSWD) of 7.72 ± 1.25 for a 30 min epoch for the entire 4 weeks period (Figure 2). A slight increase can be noticed over time which is however known to happen with aging in this strain¹⁶. Furthermore, no significant differences were found, in the four weekly EEG recordings, among the groups of WAG/Rij rats non-transplanted and those treated by antibiotics and then receiving vehicle.

The transplantation of microbiota (FMT), from the Wistar rats donors to WAG/Rij rats, significantly decreased the number and duration of SWDs ($p < 0.05$) in comparison to the aged-matched control group in the last two weeks while a non-significant decreasing effect was observable already from the second week of FMT (Figure 2). In detail, FMT significantly decreased all SWD parameters: nSWDs of ~ 45% ($p = 0.037$), dSWDs of ~ 47% ($p = 0.032$) the third week of FMT; nSWDs of ~ 45% ($p = 0.039$), dSWDs of ~ 44 % ($p < 0.022$) the fourth week of FMT; sSWD was not modified in any group.

ETH was found to be below the limit of detection (2.0 mg/l) both in the feces of donors and in the blood of recipients' rats. FMT from ETH-treated WAG/Rij rats donors was also able to decrease absence seizure parameters compared to control WAG/Rij rats similarly to what was observed after FMT from Wistar rats (Figure 2). In particular, absence seizure parameters were reduced as follows: nSWDs of ~67 % ($p = 0.021$), dSWDs of ~71 % ($p = 0.005$) the third week of FMT; nSWDs of ~69 % ($p = 0.033$), dSWDs of ~73 % ($p = 0.021$) the fourth week of FMT; sSWD was not modified in any group.

Finally, statistical analysis indicated that FMT from ETH-treated WAG/Rij rats was more effective than that from Wistar rats at both the 3rd and 4th week.

Gut microbiota manipulation in WAG/Rij rats

To clarify the contribution of gut microbiota on the absence seizure phenotype of WAG/Rij rats, the latter (6 months of age) were subjected to FMT from Wistar non-epileptic donors and Wistar rats were transplanted with the fecal microbiota from WAG/Rij rats. Microbiota composition was assessed after a 4-week FMT and results are shown by comparing FMT-treated rats to the ones receiving vehicle (see Materials and Methods, experimental protocol section). Animals were treated with an antibiotic cocktail before FMT to abrogate endogenous bacteria. Antibiotic treatment severely reduced total bacterial load, altering distribution of principal bacterial phyla (Figure S9).

Upon FMT, alpha diversity from two groups of the recipient rats was similar to that of their corresponding vehicle-treated groups (Table S2). Conversely, β -diversity analysis differentiated rats undergoing FMT in comparison with WAG/Rij and WISTAR vehicle-treated groups, respectively. Results, based on PCoA plots and ANOSIM R statistic, clearly indicated modification of both types (unweighted, Figure S10A) and the relative abundance (weighted beta diversity, Figure S10B) of bacterial species in the gut of recipient rats after FMT. Specifically, a diminution of phylogenetic distance between the gut microbiota of FMT recipients and their corresponding vehicle treated rats was observed (Figure S10A). Detailed phylogenetic analysis of the taxonomic composition of the microbiota was conducted together with SPINGO classification of significant bacterial species belonging to key genera (Table 2, Figure 3, and Figure S11). We observed that specific taxa failed to engraft in transplanted rats possibly due to host incompatibility, while others thrived (Figure 3). Particularly, statistical analysis showed that species within *Coprococcus* (*C. eutactus*) and *Phascolarctobacterium* (*P. succinatutens*) genera were significantly increased, and *Flexispira* decreased, in WAG/Rij rats after FMT from Wistar non-epileptic donors; conversely, the FMT of Wistar with bacteria from WAG/Rij rats caused a significant decrease of the relative abundance of *C. eutactus* and an increase of *Bacteroides* (*B. dorei* and *B. eggerthii*), *Parabacteroides* (*P. distasonis*) and *Akkermansia* (*A. muciniphila*) (Table 2 and Figure S11).

FMT from WAG/Rij ETH-treated rats to recipient WAG/Rij rats was also performed. As depicted in the PCoA plot, vehicle-treated WAG/Rij rats and the ETH-derived group showed non-overlapping clustering considering both type and evenness of bacterial species, indicating an effect of FMT on the remodeling of microbiota structure (Figure S10A, B). Upon FMT, significant changes were detected in relative abundance of specific bacterial taxa between the two groups (Table 2, Figures 3 and S11). In particular, members of Proteobacteria together with members of genus *Clostridium* were found decreased while *Phascolarctobacterium succinatutens* increased in FMT recipients compared to control vehicle-treated WAG/Rij rats.

Discussion

The gut microbiota, is a complex intestinal microbial ecosystem essential to health, made of bacteria, viruses, archaea and eukarya that colonizes the gastrointestinal tract. The microbiota offers many benefits to the host, through a range of physiological functions such as strengthening gut integrity or shaping the intestinal epithelium²⁶, harvesting energy²⁷ and regulating host immunity²⁸. Many studies demonstrate a microbiota-gut-brain bidirectional connection *via* neural, endocrine, metabolic and immune pathways^{29,30}. It was shown that, gut microbiota alterations, may have impact on these pathways that contribute to neuronal hyper-excitability and neuro-inflammation in epilepsy and in other neurodegenerative diseases. Recent studies examined the effects of a ketogenic diet in two mouse models of refractory epilepsy; gut microbiota (and particularly *Akkermansia* and *Parabacteroides*) was found to be necessary for the diet to effectively reduce seizures³¹. In other studies, changes in the gut microbiota of children with drug-resistant epilepsy were found in comparison to healthy children. The ketogenic diet significantly improved the gut microbiota (increased Bacteroidetes and decreased Firmicutes and Proteobacteria) with a decrease in seizure frequency in epileptic children^{32,33}, although the specific role of alterations in selected bacteria phyla has to be confirmed. Accordingly, it was recently demonstrated that dysbiosis associated with chronic stress enhances kindling epileptogenesis, and FMT from sham-stressed animals transplanted to chronically stressed rats counteracts proepileptic effects of restraint stress³⁴. Moreover, probiotic supplementation (*Lactobacillus rhamnosus*, *Lactobacillus reuteri*, and *Bifidobacterium infantis*) reduced seizure severity in the pentylenetetrazole (PTZ)-kindling model as well as improving spatial learning and memory in the Morris water maze test along with increased GABA levels³⁵; this latter result is in agreement with a pilot study with probiotic supplementation (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus brevis*, *Bifidobacterium lactis*, and *Streptococcus salivarius* subsp. *thermophilus*) in epileptic patients³⁶. Microbial products act as signaling molecules that can impact directly or indirectly on the CNS and the enteric nervous system. Gut microbiota alteration may increase inflammatory cytokines and

bacterial metabolites may alter the gut- and blood-brain barriers permeability causing neuroinflammation³⁷; however, several other mechanisms may be involved in this not yet completely explored link^{9,38}.

Our results, for the first time, demonstrate that in a genetic animal model of epilepsy, the gut microbiota is early altered even before seizure appearance, that this alteration is maintained over time and that microbiota manipulation can reduce seizure occurrence. Furthermore, we observed that microbiota alterations are also accompanied by structural inflammatory modifications of the ileum but minimally of the colon.

WAG/Rij rats display a specific modification of the gut microbiota composition already at the age of 1 month when absence seizures are not yet developed, and this is characterized by a lower Bacteroidetes/Firmicutes ratio ~~due to an increase in Firmicutes~~ with respect to control Wistar rats. This difference is likely linked to the genotype and is, already at this age, accompanied by inflammatory intestinal damage but not yet increased intestinal motility. In this case, intestinal damage may be a consequence or a cause of the altered microbiota, however alterations of the intestinal barrier together with the underlying inflammation may contribute to the WAG/Rij phenotype. Bacteroidetes and Firmicutes strongly influence the final levels of intestinal SCFAs (e.g. propionate and butyrate) and their observed reduced concentrations could result from reduction of fermenting bacteria of the Bacteroidetes phylum and imbalance of specific members of Firmicutes. Indeed, absence seizure improvement in older rats was accompanied by a recovery of intestinal morphology in our experiments and intestinal inflammation is known to increase seizure susceptibility^{5,6}. FMT from WAG/Rij rats to Wistar rats induced intestinal damage, suggesting a direct effect of WAG/Rij rat gut microbiota on intestinal structure. Specifically, we observed a concomitant overgrowth of *Akkermansia muciniphila*, whose role is currently under study in neurological diseases such as ASD, PD, MS and AD where increased abundance of this species has been reported³⁹⁻⁴². Despite this mucin-degrading intestinal bacterial species has been considered a potential probiotic, due to its protective effect in many metabolic disorders⁴³, high levels of A.

muciniphila have been associated to reduced intestinal mucus thickness, increased gut permeability and inflammation⁴⁴ possibly promoting systemic inflammation and brain damage. Thus, the hypothesis of common etiological mechanisms for neurological diseases of gastrointestinal derivation deserves further exploration in order to assess the role of microbiota and whether microbiota analysis could be used as a biomarker for several purposes (*e.g.* diagnosis, prognosis etc)^{9,10}.

Our data also showed a much more marked differentiation later in development, in 4 month old WAG/Rij rats already displaying a high number of absence seizures per day paralleling a modification of gut microbiota; a further reduction in the Bacteroidetes/Firmicutes ratio was observed (which is also physiologically happening in Wistar rats) and a strong reduction of Bacteroidetes *vs* Firmicutes was evidenced along with an increase in members of Proteobacteria and Tenericutes. Moreover, a prominent reduction of several species of *Lactobacillus* was observed in WAG/Rij with respect to Wistar rats, according to their beneficial effects on epileptic phenotype^{35,36} This fingerprint gut microbiota composition is accompanied by a reduction in the production of SCFAs which are known to be protective at the intestinal level but also to act as messengers between the gut and the brain⁴⁵; furthermore, it is known that butyrate has some antiabsence effects in this strain of rats⁴⁶. On the other hand, we report a proliferation of specific taxa within Bacteroidetes and Proteobacteria producing lipopolysaccharides and possibly increasing inflammation at the intestinal level and leading to systemic and central inflammatory signals which, in turn, could modulate seizure susceptibility in this animal model^{9,10,16}.

The proof of the relevant role of gut microbiota on absence seizures in this model and intestinal damage is demonstrated by our results after FMT. This latter treatment is the process of transplanting fecal bacterial communities from a healthy donor to a recipient whose microbiota has been disrupted or altered to cure a specific disease⁴⁷ such as in the case of treating refractory and recurrent *Clostridium difficile* infection (CDI)⁴⁸. He et al., reported the first case of FMT to achieve remission of intestinal and neurological symptoms in a girl with Crohn's disease and a 17-year history of epilepsy⁴⁹. Similarly, FMT, from sham-stressed animals transplanted to chronically stressed rats,

counteracted the proepileptic effects of restraint stress³⁴, as above mentioned. Here, FMT from non-epileptic Wistar rats to WAG/Rij rats caused a reduction of absence seizures and partial recovery of intestinal structure in 4 weeks. This was accompanied by a reshaping of gut microbiota with a reduction in the phylogenetic distance between their respective controls; accordingly, FMT from WAG/Rij to Wistar rats resulted in intestinal damage driven by the implanted gut microbiota. Surprisingly, FMT from WAG/Rij rats treated with a high dose of ETH (nearly abolishing all seizures over 24 hours) also reduced absence seizures in recipient rats and this effect was also accompanied by a reshaping of gut microbiota. Undoubtedly, ETH is not present in the feces considering its nearly complete bioavailability and therefore this effect must be due to the modification in microbiota composition. Of note, the modification in gut microbiota composition is different when considering FMT from Wistar or ETH-treated rats if not for the substantial increase in the presence of *Phascolarctobacterium succinatutens*. This latter organism is known to convert succinate to propionic acid and both have been suggested as messengers inside the GMB axis⁵⁰; however, their role in absence seizures and epilepsy should be further investigated and other studies are needed in this model to understand which may be the most important mediators involved in the link between gut microbiota and epilepsy. In any case, the positive effect of FMT from ETH-treated rats also suggests that part of ETH action may be mediated by its effects on gut microbiota and, although no data in this direction are available, ETH side effects on the gastrointestinal tract are well known and may share common mechanisms; indeed, ETH has been reported to modify intestinal motility which may *per se* have an effect on gut microbiota composition⁵¹.

Overall, our results demonstrate that the gut microbiota is involved in absence seizures in the WAG/Rij rat model, extending our knowledge in this intriguing research area. The availability of animal models may also facilitate the study of the GMB axis and would define whether it can be used for the several purposes so far supposed (*e.g.* a treatment target as well as a biomarker). Finally, its manipulation seems promising; however, many variables may count and further research is warranted. In conclusion, WAG/Rij rats represent so far, the first genetic animal model of epilepsy

with an established involvement of gut microbiota and many other models should be investigated in the future in order to extend our knowledge.

Key Points Box

- Gut microbiota is altered in the WAG/Rij rat genetic model of absence epilepsy
- Manipulation of gut microbiota by fecal microbiota transplant reduces absence seizures in WAG/Rij rats
- Gut microbiota and intestinal alterations precede absence seizure onset in WAG/Rij rats

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Disclosure of Conflicts of Interest

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Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Figure legends

Figure 1. Gut microbiota composition at phylum level in WAG/Rij rats over time. A) Heatmap of most counted OTUs (relative abundance > 0.5%) from WAG/Rij and Wistar rats at 1 month, 4 months and 8 months of age. Each column represents an individual rat microbiota and each row an OTU. Rows and columns are sorted by group or phylum-level taxonomic assignment, respectively. B) Pie charts showing the relative abundance of all bacterial OTUs taxonomically classified at phylum level. C) Ratio of *bacteroidetes* to *firmicutes* (mean \pm SEM) in each sample group; data with different superscript letters are significantly different at $p < 0.05$, according to the one-way ANOVA followed by Tukey's multiple comparison *post-hoc* test.

Figure 2. Effects of fecal microbiota transplantation (FMT) on spike-wave discharge number (nSDWs) and duration (dSWDs) on electroencephalographic (EEG) recordings in WAG/Rij rats. Rats were repeatedly EEG recorded PRE-ABX treatment, at the POST-ABX treatment and then weekly during FMT. This latter treatment from both Wistar donors or ethosuximide (ETH)-treated WAG/Rij rats induced a significant reduction (*; $p < 0.05$) in nSWDS and dSWDs in comparison to vehicle-treated and control rats. Interestingly, ETH-FMT was significantly (#; $p < 0.05$) more efficacious than Wistar FMT.

Figure 3. Selection of bacterial taxa in FMT-treated rats. Heatmap graph of one-way ANOVA analysis of microbiota profile up to genus level with relative abundance >0.5% in at least one of the groups (rows) of FMT and vehicle-treated rats (columns). * highlights the fraction of taxa significantly different between transplanted rats and the corresponding vehicle group (same genotype); # highlights the fraction of taxa significantly different between transplanted and vehicle rats (different genotype).