UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF
Neurosciences, Biomedicine and Movement Sciences

GRADUATE SCHOOL FOR
Life and Health Sciences

DOCTORAL PROGRAM IN
Neuroscience, Psychology and Psychiatry

Cycle / year
XXX/2014

DOCTORAL THESIS

Possible impact of the gut microbiota on the excitability of the brain

Coordinator: Prof. Leonardo Chelazzi

Tutor: Prof. Paolo F. Fabene

Doctoral Student: Dr. Inna Kosenkova

Verona 2018

List of abbreviations

ACTH	adrenocorticotropic hormone
BBB	blood–brain barrier
CNS	central nervous system
EEG	electroencephalography
ENS	enteric nervous system
ERPs	event-related potentials
FMT	fecal microbiota transplantation
GALT	gut-associated lymphoid tissue
GBA	gut-brain axis
GF	germ free
GIT	gastrointestinal tract
GM	gut microbiota
HPA	hypothalamic-pituitary-adrenal axis
ILAE	International League Against Epilepsy
IPANs	intrinsic primary afferent neurons
IS	ischemic stroke
KD	ketogenic diet
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
MSC	methyl scopolamine
MRI	magnetic resonance imaging
nt	non-target stimulus
NVU	neurovascular unit
OTU	operational taxonomic unit
SCFAs	short chain fatty acids
SE	status epilepticus
SPF	specific pathogen free
SRSs	spontaneous recurrent seizures
TLE	temporal lobe epilepsy
t	target stimulus
VN	vagus nerve
WHO	World Health Organisation

Abstract

It is becoming increasingly evident that the role of the gut microbiota (GM) is not limited by the walls of the gastrointestinal tract (supporting the digestion, absorption of nutrients, intestinal motility and resistance to pathogens), but it also influences normal physiology of the whole organism and contribute to the broad range of diseases including those affecting the central nervous system (CNS).

The growing appreciation of the role of intestinal bacteria in brain physiology has led to the establishment of so called “gut-brain axis”, or the “microbiota-gut-brain axis”, a bidirectional communication network between the gut and the brain.

The exact pathways of interaction are still being revealed and they include: immune pathway (immune signalling cells, cytokines, chemokines), endocrine pathway (HPA axis), neural mechanisms (vagus nerve and enteric nervous system) and metabolites: bacterial-derived cell wall components (peptidoglycans) and bacterial-derived metabolites, such as SCFAs.

It has been reported, that gut microbiota can have an effect on brain function altering the levels of circulating cytokines. Recent studies consider that abnormal levels of cytokines/chemokines and immune cells play an important role in the epileptogenesis process. Furthermore, increase in cytokines has been reported to play a neuromodulatory role. The immune-mediated leakage in the blood–brain barrier (BBB) was showed to be involved in both, the induction of seizures and the progression to epilepsy. Moreover, the gut microbiota may also influence BBB permeability in mice and commensal intestinal microbiota was found to be able to modulate excitability of gut sensory neurons.

Summarizing above mentioned, we hypothesized that gut microbiota form subjects affected by neural pathology can modulate in healthy subjects excitability in CNS and, finally, positively correlate with the level of seizure activity.

The primary aim of the research project was to determine the impact of microbiota transplanted from animals with induced epilepsy to healthy recipient animals, in order to clarify whether the presence of the disease can change microbiota in the gut to the point of having a “pro-pathological” microbiota.

The data obtained in this study suggests that mice received “pro-pathological” microbiota have compromised brain excitability. Microbiota composition of the donors with induced temporal lobe epilepsy (TLE) was characterised by the increase in *Sutterella, Prevotella, Dorea, Coprobacillus* and *Candidatus Arthromitus* in comparison with the baseline. These alterations, through the GBA, may possibly have an effect on the excitability of the brain and subsequently on the threshold for the seizure activity.

Introduction

Microbiota definition

Humans through their lifetime live in cooperation with the huge amount of microorganisms inhabiting body cavities and all exposed and internal surfaces of the body.

This community represents a complex ecosystem which is formed not only by microbes, but it includes bacteria, archaea, protozoa, fungi and nematodes (mostly parasitic).

All collection of the microorganisms in the community is termed microbiota, and the collection of their genomes - microbiome. These microorganisms occupy distinct microbial niches where they perform tissue-specific functions and each of these niches may have a different composition of microorganisms. Among others, the most studied consortia are: oral microbiome (Eren, Borisy, Huse, & Mark Welch, 2014), vaginal (van de Wijgert et al., 2014), microbiome of the skin (Weyrich, Dixit, Farrer, Cooper, & Cooper, 2015), nasal and sinus microbiome (Wilson & Hamilos, 2014).

Although microbiota composition varies a lot across body niches and time, corresponding bacterial communities across individuals tend to be more similar to each other (Costello et al., 2009). The quantitative characterization of the microbiota is still under the discussion. For the last four decades, one of the most cited and exalting paradigm was that “microbial cells outnumber human cells 10 times”, “we are only 10%” human” etc. (Luckey, 1972). Recently the amount of human and bacterial cells in the body was critically recalculated and the new ratio of human/bacterial cells representation was established = 1.3:1 (Sender, Fuchs, & Milo, 2016).

At the same time, last studies confirm that the most densely inhabited organ, in terms of microbiota, is colon (concentration of microbial cells there arrives at $10^{14}/cm^3$) making it the "only significant contributor within the all gastrointestinal tract". For comparison a stomach has 10^3 cells/cm^3, duodenum 10^3-10^4, ileum 10^8 (Table 1).

Table 1. Bounds of bacteria number in different parts of the gastrointestinal tract (GIT).

Part of digestive system	Typical concentration of bacteria (number/ml content)	Volume (ml)	Order of magnitude bound for bacteria number
stomach	10^3 - 10^4	250-900	10^7
duodenum and jejunum	10^3 - 10^4	400	10^{11}
ileum	10^8		
colon	10^{11}	420±90	10^{14}

Modified form (Sender et al., 2016)

The total weight of the intestinal microbiome arrives to 1-2 kg, making it comparable with the weight of the human brain (Stilling et al., 2014); it represents a complex endocrine organ seems to be so crucial in the wellbeing of the host that it was already named as "second brain" (Gershon, 1999) or even as the "forgotten organ" (O'Hara & Shanahan, 2006).

The experiments that will be described in this thesis are focused in this particular microbial niche, thus, from here, by the term of "microbiota" only intestinal community of the microbiota will be intended.

Intestinal microbiota

Microbiota community in the human gut is mainly composed of strict anaerobes and is formed by nine divisions of bacteria: *Bacteroidetes and the Firmicutes* that are dominating and compose more than 98% of the total volume and *Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, Cyanobacteria*, *Spirochaeates* and *VadinBE97* that are present in a smaller proportions. Mice showed similar patterns in the microbiota composition and representation of the bacterial phyla, with the exception that *Fusobacteria* and *TM7* were not detected (Ley, Peterson, & Gordon, 2006).

Gut microbiota has been already recognized for the 'in-field' functions, closely connected to the physiology of the GIT, where it is responsible for the digestion and absorption of nutrients, fat distribution, synthesis of vitamins (K, B12), intestinal motility and gut barrier homeostasis (Bäckhed et al., 2004; Bercik, Collins, & Verdu, 2012).

Nowadays it is becoming increasingly evident, that the role of the gut microbiota is not limited by the walls of the GIT, but it also contributes to normal physiology of the whole body playing role in metabolism, maturation and activity of the immune system and behavior of the host (Barko, McMichael, Swanson, & Williams, 2017).

Moreover, in case of dysbiosis it was shown to contribute to the broadest range of diseases. Some, the most studied to date are: obesity, inflammatory bowel disease, metabolic syndrome, cardiovascular diseases (Cho & Blaser, 2012), immune-Mediated Inflammatory Diseases such as rheumatoid arthritis and psoriasis (Forbes, Van Domselaar, & Bernstein, 2016) and the uprising evidence also links intestinal to diseases of the CNS.

Relation of Intestinal microbiota with CNS

Emerging data suggest that gut microbiota has an ability to influence psychological state of the host.

Many behavioral alterations were observed in germ free animals (GF) - those free from microbial colonization and kept under strictly sterile conditions.

Experiments revealed deviations in the psychological state of the sterile animals compared to the conventional mice: they were reported to experience reduced anxiety-like behaviour showing more risk-taking activity, increased sociability, lack of normal stress responsivity and learning and memory deficits. Interestingly, in many cases recolonisation of the GF mice with the conventional microbiota leaded to normalisation of behavior manifestations and responses. (Clarke et al., 2013; Diaz Heijtz et al., 2011; Gareau et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011).

Moreover, the composition of the intestinal microbiota has been already linked to the pathogenesis of numerous severe neurological diseases including autism spectrum disorder (Kang et al., 2013; Wang et al., 2011), schizophrenia (Dinan, Borre, & Cryan, 2014) and multiple sclerosis (Cantarel et al., 2015; Freedman, Shahi, & Mangalam, 2017). The importance of GM alteration was also demonstrated in Parkinson's disease (Scheperjans et al., 2015), Alzheimer's disease (Mancuso & Santangelo, 2017; Zhao, Jaber, & Lukiw, 2017) and stroke outcome (Benakis et al., 2016; Winek, Engel, et al., 2016).

Again, many insights on the involvement of the GM in the brain functioning were brought up through the studies performed on the GF animals. Mice with

the lack of microbes were reported to have increased blood-brain barrier BBB permeability (reversed by the colonisation of these animals with the normal microbiota) (Braniste et al., 2014), hypermyelination of the prefrontal cortex that is also reversible by colonization with a conventional microbiota (Hoban et al., 2016).

Gut-Brain Axis

Rapidly increasing appreciation of intestinal bacteria in shaping brain development and behaviour has led to the establishment of so called "gut-brain axis" (Foster & McVey Neufeld, 2013), which later was enlarged to the "microbiota-gut-brain axis" - especially underlining the importance of the gut microbiota in this alliance (Mayer, Tillisch, & Gupta, 2015). But still in the scientific literature gut-brain axis (GBA) is more common abbreviation, which, however, still involves intestinal microbiota as an important player in this field.

The GBA is a bidirectional communication network between the gut and the brain. This interaction is maintaining through the mechanisms that are complex and they are still being revealed. To this moment, data accumulating both from animal research models and clinical practice suggest the presence of several pathways: neural, hormonal, immune pathways, and via neurotransmitters.

Neural pathway, including the Vagus nerve (VN) and Enteric Nervous System (ENS). The VN (cranial nerve X) is recognized as a direct connection between intestine and the brain having both efferent and afferent roles. Experiments on the vagotomized animals showed that certain strains of bacteria can modify stress response, anxiety, depression and cognition of the host and these effects are dependent on the vagus nerve, identifying it as a major pathway between the bacteria inhabiting the gut and the brain (Bravo et al., 2011).

In addition to this direct communication, vagal-independent interaction also takes place. Microbiota constantly directly interacts with ENS – the largest part of the autonomic nervous system which contains approximately 50,000 extrinsic sensory neurons and 100 million intrinsic afferent neurons (Blackshaw, Brookes, Grundy, & Schemann, 2007; Furness, 2006) responsive to bacteria. They include motor neurons, interneurons and intrinsic primary afferent neurons (IPANs) which detect mechanical and chemical stimuli directly from the lumen. (Nagy & Goldstein, 2017). However, mechanisms by which ENS can modulate GBA are currently unclear.

Hormonal pathway is working through the hypothalamic-pituitary-adrenal (HPA) axis - crucial system for providing a physiological adaptation of the organism to the stress (Herman et al., 2016). The HPA axis regulates the secretion of cortisol. This stress-related hormone can change gut barrier homeostasis and microbiota composition ant alter gut permeability (O'Mahony, Hyland, Dinan, & Cryan, 2011). Alterations of the microbiota composition have been already widely associated with the stress, especially if occuring in the early life. (De Palma et al., 2015; Golubeva et al., 2015; Sudo et al., 2004). Maternal separation in rats was reported to alter the microbiota composition in pups (O'Mahony et al., 2009). Experiments comparing a GF mice with a conventional mice revealed basal differences in biomarkers related to the stress response and showed that after the restraint test animals who had no exposure to the microorganisms had higher level of the adrenocorticotropin hormone (ACTH) in plasma and corticosterone elevation. What is more, this response in GF mice was reversed by recolonization with *Bifidobacterium infantis* (Sudo et al., 2004).

Apart from the changes in the composition of the microbiota under the condition of stress, the HPA axis can be also influenced by the bacterial-derived metabolites, such as short chain fatty acids (SCFAs), produced by fermentation of the dietary fibers by intestinal bacteria. SCFAs, by binding to the intestinal G-coupled receptors, were shown to induce the secretion of the hormones from enteroendocrine cells (Samuel et al., 2008).

Taken together, exact mechanisms by which endocrinology-based mechanisms are taking part in microbiota - brain interaction have not been elucidated yet, but the evidence from the stress-related research suggest this pathway as a potential target for the regulation of the GBA.

Immune pathway (immunal signalling molecules, cytokines, chemokines)

It was shown that intestinal microbiota is required for the normal development and maturation of the innate and adaptive immune systems (Chow, Lee, Shen, Khosravi, & Mazmanian, 2010). Later on, both these systems are involved in maintaining homeostasis on the luminal surface of the intestine (the primary interface between the microbiota community and internal host tissues), what is crucial for maintaining health of the host (Duerkop, Vaishnava, & Hooper, 2009).

Also in this case, studies in a GF mice reveal significant deviations in comparison to the animals housed under specific pathogen free conditions. A GF animals appear to have fewer and smaller mesenteric lymph nodes and Peyer's patches, and deviations in the composition of the diffuse gut-associated lymphoid tissue (GALT) - primary components for immune response against pathogens. Notably, the number of the intraepithelial lymphocytes was increased after the colonization of GF rodents with an intact

microbiota (Falk, Hooper, Midtvedt, & Gordon, 1998; Kwa, Beverley, & Smith, 2006; Round & Mazmanian, 2009).

Intestinal immune homeostasis is maintained through the balanced communication between pro- and anti-inflammatory mechanisms affected by the microbial activity in the gut.

Emerging data suggest that intestinal microbiota can influence the alteration of circulating levels of inflammatory and anti-inflammatory cytokines, chemokines and immune signaling molecules (Alam, Abdolmaleky, & Zhou, 2017) and modulate low-grade inflammation both in the gut and systemically (Cryan & Dinan, 2012; Pisanu & Squassina, 2017).

Influence the level of neurotransmitters

Some bacteria were shown to have a capacity to synthesize and release several neurotransmitters and neuromodulators: for example, *Candida spp.*, *Streptococcus spp*. and *Escherichia spp*. act in serotonin production, *Bacillus spp*. can produce dopamine, some species of *Lactobacillus* - acetylcholine, *Escherichia spp*., *Bacillus spp.* and *Saccharomyces spp*. produce noradrenaline. One of the most important inhibitory neurotransmitters - gamma-aminobutyric acid - can be produced by certain species of *Lactobacillus* and *Bifidobacterium*.

It was proposed that neuroactive metabolites produced by the microbiota are important not only for the interaction with the host, but also as a way of communication between members of the microbiota community (Lyte, 2010).

Conclusion:

Constant functioning interaction between the microbiota, gut and the brain, either directly via vagus nerve and microbial metabolites or indirectly through endocrine and immune pathway appears to be crucial for the normal

physiology of the host. Further studies are needed to elucidate the numerous unrevealed factors in this complex communication network.

Temporal Lobe Epilepsy

Epilepsy is a life-threatening progressive neurological disorder characterized by uncontrolled abnormal brain activity clinically manifesting as recurrent unprovoked seizures.

It affects 50-65 million people worldwide. Estimated 0.5-1% of the population in developed countries suffer from this disease, and almost 2 times more in the developing. And each year 2.4 million people are becoming diagnosed with epilepsy. These numbers become even more dramatic when we face the fact that about a third part of all the cases are still considered to be incurable and patients can undergo only neurosurgery, symptomatic treatment or other beneficial therapy such as neurostimulation or special diet, which, however, may not guarantee a seizure-free life (Moshé, Perucca, Ryvlin, & Tomson, 2015; “WHO | Epilepsy,” 2017).

Approximately 60% of all people living with epilepsy are diagnosed with the temporal lobe epilepsy (TLE) - the most frequent type of focal (partial) epilepsy and, regrettably, the most common cause of drug-resistant seizures. Even though the antiepileptic drugs are affordable and inexpensive to control or to reduce seizure occurrence, but approximately one third of patients remains resistant to pharmacological treatment (Löscher & Potschka, 2005; “WHO | Epilepsy,” 2017). Epilepsy has no geographical, racial or social boundaries and it remains one of the biggest challenges in the neurological clinical practice and research.

There are two types of the TLE. The first, most common one, is medial TLE, it involves the medial or internal structures of the temporal lobe; while the second, called neocortical TLE, involves the outer portion of the temporal lobe. In clinical practice symptoms of TLE are manifesting as seizures that originate from the hippocampus, entorhinal cortex or amygdala that can be developed many years after SE. Diagnosis is based mainly on the results from the electroencephalography and magnetic resonance imaging.

Involvement of Neurovascular unit in pathophysiology of Epilepsy

An epileptic seizure, as defined by the ILAE “a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain” results from the abnormal synchronization of the neurons that disturbs the physiological excitation and inhibition balance and disrupts neuronal communication (Fisher et al., 2017).

For many years epilepsy was considered as a “neuron-firing” disease, making affected neurons the most important contributors to the pathogenesis. Recent studies revealed that in fact the neuronal activity depends on the strong interaction between neurons, glial cells, BBB permeability, vascular endothelium, humoral network (including cytokines and chemokines) and also non-neuronal immune-related blood cells; what is rising a new paradigm considering this integrated system of the neurovascular unit (NVU) as an extremely important complex for the understanding pathogenesis of epilepsy (Bertini et al., 2013).

The NVU represents a concrete anatomical and functional structure including: neurons, the BBB, glial cells, pericytes, leukocytes, the humoral network: cytokines, chemokines and extracellular matrix.

Each of these components contribute to the pathogenesis of epilepsy in direct and indirect ways: BBB damage was reported to be “a hallmark of seizure activity”, and it is involved both in the induction of seizures and progression to chronic state of disease (Marchi et al., 2010; Marchi & Lerner-Natoli, 2013). Pericytes were shown to modulate BBB integrity (their deficiency is leading to leaky BBB) and now they seem to have an important role in the activity of the epileptic brain (Milesi et al., 2014).

Accumulating data both, from research models and clinical practice, suggest that also leukocytes, especially leukocyte-endothelial adhesion mechanisms, have an important role in the pathogenesis of epilepsy. It was shown that leukocyte trafficking mechanisms provoke BBB leakage which as it was already mentioned is leading to the seizure activity. Recent studies revealed that blockage of leukocyte-vascular adhesion prevented BBB disruption in animal model of epilepsy (Fabene et al., 2008). This is supported with the insights from the clinical practice: patients with different types of epilepsy reported to have more leukocytes in brain parenchyma in comparison with controls (Fabene, Laudanna, & Constantin, 2013).

Closer look to the humoral network, another component of the NVU, can help for better understanding of the seizure origins. Here, chemokines - small secreted proteins that primarily control the migration and activation of the immune cells play role. Recently, chemokines, besides their immunological competence, were shown to have neuromodulator and neurotransmitter-like effects (Stuart & Baune, 2014) and to be able to modulate the above mentioned mechanism of the leucocyte-vascular adhesion. Another players of the humoral network are cytokines, small soluble mediators that are crucial in immune regulation. They appear to modulate the thalamo-cortical synchronisation: for example, it was shown that pro-inflammatory cytokine IL-1β have a pro-convulsant effect (Vezzani et al., 2002).

Thus, the NVU represents a complex system deeply involved in the brain physiology and pathological states including epilepsy.

Role of inflammation in pathogenesis of epilepsy

The concept of neurovascular unit as a complex integrated system is important for introducing one of the recently emerged factors of epileptogenesis - inflammation. Growing body of literature suggests that inflammatory mechanisms have an important role in the development, progression and occurence of the seizure activity. What is more, seizures *per se* can induce inflammation of the brain and recurrency is leading to the chronic inflammation (Vezzani, French, Bartfai, & Baram, 2011).

From the clinical practice it was reported that prolonged or focal febrile seizures (those induced by fever) in childhood have been associated with the occurrence of intractable form of the TLE epilepsy (Dubé, Brewster, Richichi, Zha, & Baram, 2007). Also, human brain tissue obtained during the surgical resection of the seizure focus in patients with refractory focal epilepsy appeared ho have significantly more biomarkers of inflammation than healthy individuals (Vezzani et al., 2011).

In vitro and *in vivo* research models offer a huge advantage for the better investigation of the inflammatory process that can trigger (or be triggered by) seizure activity. Available studies highlight several mechanisms that contribute to the epilepsy-related inflammation. These are: neuroinflammation, vascular inflammation, BBB leakage, recruitment of inflammatory cells, leukocyte-endothelial adhesion, etc. Inflammatory mediators were also shown to be involved (Bertini et al., 2013).

Thus, evidence from the clinical practice and numerous animal models suggest that inflammation might be both a consequence and a cause of epilepsy.

Hypothesis Gut microbiota-Inflammation-Epilepsy

Based on the previously reviewed data:

- Abnormal levels of cytokines/chemokines and immune cells were reported to have an important role in epileptogenesis (Fabene, Bramanti, & Constantin, 2010; Li et al., 2011).

The immune-mediated leakage in the BBB is involved in both, the induction of seizures and the progression to epilepsy (Fabene et al., 2008).

And on the other "side":

- Gut microbiota can have an effect on brain function altering the levels of circulating cytokines (Cryan & Dinan, 2012).
- The GI represents a vulnerable surface through which pro pathogenic microorganisms can influence various aspects of physiology and cause inflammation in the central nervous system (Catanzaro et al., 2015).
- New evidenced are emerging on the fact that the commutal intestinal microbiota is able to modulate the excitability of intestinal sensory neurons (Neufeld et al., 2011).

Summarizing the above mentioned data, we hypothesized that gut microbiota from subjects affected by neural pathology can modulate in healthy subjects excitability in CNS and finally positively correlate with the level of seizure activity (Fig. 1).

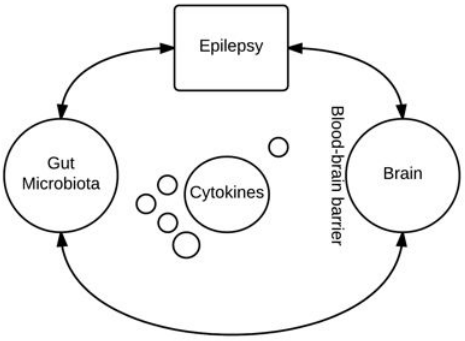

Figure 1. Hypothetical relation of GM with brain excitability via inflammatory pathways.

Experiments

Part I. Establishment of the experimental groups of the microbiota donors

Rationale

Changes in the microbiota composition were shown to be associated with several neurological pathologies. The present project aims to (i) examine an impact of pathological conditions of the nervous system (chronic epilepsy) on the composition of gut microbiota and (ii) the ability of the pro-pathological microbiota to influence the excitability of the brain through lowering the threshold of the seizure activity.

The primary aim of present study is to establish and describe experimental groups of microbiota donors and create a biobank with the fecal microbiota samples for the next step inoculations.

Experimental design

Pilocarpine mouse model of the TLE

Among all the types of epilepsy, temporal lobe epilepsy has one of the highest representation of the therapy-resistant cases. The patho(physio)genesis of TLE is still being revealed and the use of animal models are essential to discover the exact pathways.

In clinical practice TLE is usually characterized with the localization of seizures in the limbic system, particularly in the hippocampus, entorhinal cortex and amygdala (Bartolomei et al., 2005); “initial precipitating injury” which appears before the diagnosis of TLE (Mathern, Adelson, Cahan, & Leite, 2002); a

seizure-free period known as “latent” or “silent” period; and frequently observed presence of the hippocampal sclerosis.

Most of the above mentioned features can be reproduced in mouse model by the systemic injection of the muscarinic cholinergic agonist - pilocarpine hydrochloride (Curia, Longo, Biagini, Jones, & Avoli, 2008).

Administration of the drug is leading to *status epilepticus* (SE) development, after a latent seizure free period, to spontaneous recurrent seizures (SRSs) occurrence and resulting in a chronic condition of epilepsy (Navarro Mora et al., 2009). Thus, the pilocarpine model of epilepsy in mice is by now one of the most studied, well-characterized and widely used in laboratory practice since it was first described in 1983 (Turski et al., 1983).

The dosage and the way of the drug-administration can be modified depending on the aims of the experiment.

For this work we based on the optimal conditions proposed by Mazzuferi et al. (Fig. 2a) that are producing the highest response (50%) associated with the best survival rate of the animals (90%) (Mazzuferi, Kumar, Rospo, & Kaminski, 2012).

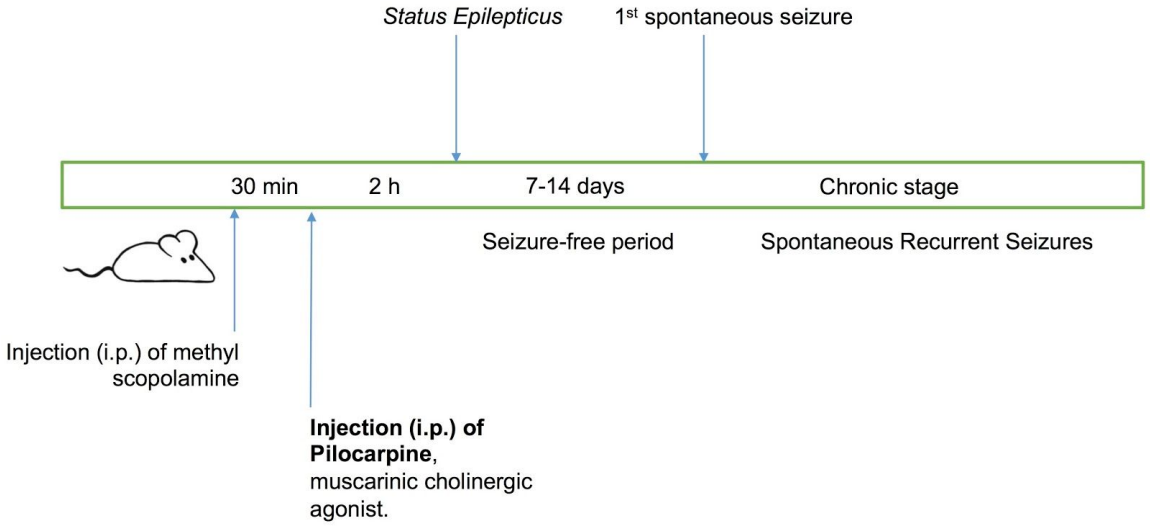

Figure 2a. Timeline of TLE mouse model used in the experiments.

General description

To induce epilepsy and establish experimental groups NMRI mice were injected with the corresponding drugs (Fig. 2b). Only male mice were used, as both from clinical practice in women and laboratory practice in female rodents, gonadal hormones (estrogen, progesterone) were reported to deeply affect neuronal excitability and the physiological response for the pilocarpine-induced SE (Scharfman et al., 2005; Scharfman & MacLusky, 2006).

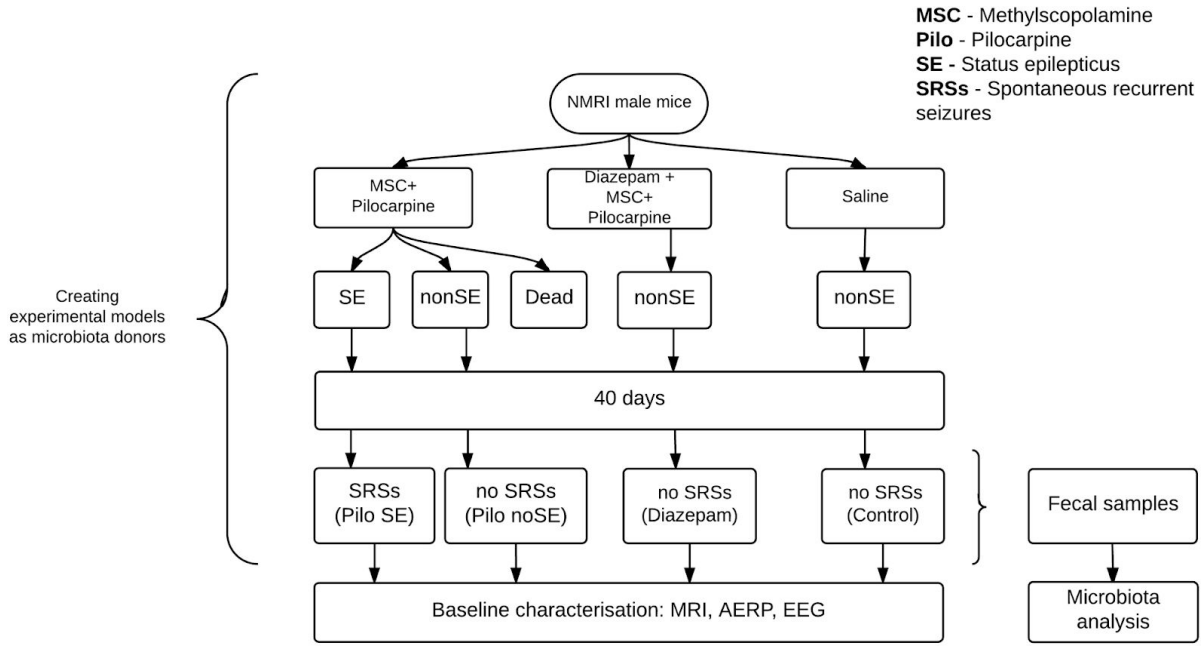

Figure 2b. Flowchart of the experiment aiming to establish groups of “pro-pathological” microbiota donors.

The first group of animals were injected according to the pilocarpine model for the TLE.

Before the pilocarpine mice were injected with the methylscopolamine to minimize peripheral cholinergic effects (Curia et al., 2008).

The second group was pre-treated with the bolus dose of the Diazepam – positive allosteric modulator of the $GABA_A$ chloride channel which was

reported to have anticonvulsant properties and which is broadly used to terminate the convulsive SE (Calcaterra & Barrow, 2014).

Pre-treatment with Diazepam in our case has the role of inhibiting the development of pilocarpine-induced seizures, providing also the neuroprotective effect (Radzik, Miziak, Dudka, Chrościńska-Krawczyk, & Czuczwar, 2015).

The third group was the naïve controls. Detailed protocol of injections is reported in the 'Materials & Methods' section.

Next, the 40 days after drugs injections fecal samples were collected to create a biobank for the next experiments considering fecal microbiota transplantation (FMT). Circadian rhythm has been shown to have an impact on the composition of the murine microbiome (Liang & FitzGerald, 2017). Thus, all the sampling procedures were performed in the dark phase from 9:00 am till 11:00 am.

Materials and Methods

Animals

NMRI male mice (Charles River, France) 7 weeks old (weighing 32-38g) were used. After injections animals were kept 1 per cage under controlled environmental parameters (inverted 12:12 light-dark cycle, temperature 22 ± 1°C, humidity 50–60%, food and water *ad libitum*) in Optimice® cages (Animal Care Systems Inc., Centennial, USA).

Animal care and experimental procedures were conducted in accordance with the guidelines of the European Union directive 2010/63/EU and all protocols were approved by local ethical committee (Centro Interdipartimentale di Servizi per la Ricerca che utilizza Animali da Laboratorio – C.I.R.S.A.L., University of Verona) and Italian Ministry of Health (Authorisation №.1107/2015-PR).

Ethical issue

The pilocarpine murine model for the study of temporal lobe epilepsy is widely used and accumulated knowledge within the scientific community allows to design the experiment minimizing the number of animals and maximizing the obtained results.

Furthermore, studies must be done *in vivo* to have an ability to observe changes in brain function and behavior manifestations.

Induction of *Status epilepticus* and epileptogenesis

Status epilepticus (SE) was elicited by the intraperitoneal injection of pilocarpine (Sigma, 300 mg/kg, i.p). In order to reduce the peripheral cholinergic effects of pilocarpine (PILO) animals were injected with a scopolamine methyl nitrate (Sigma) in a dose 1 mg/ kg, i.p 30 minutes before PILO. Additional group of animals received a bolus dose of Diazepam (10 mg/kg, i.p) twenty minutes prior to methyl scopolamine. Control group was formed by mice injected with the corresponding volumes of saline (Fig. 3).

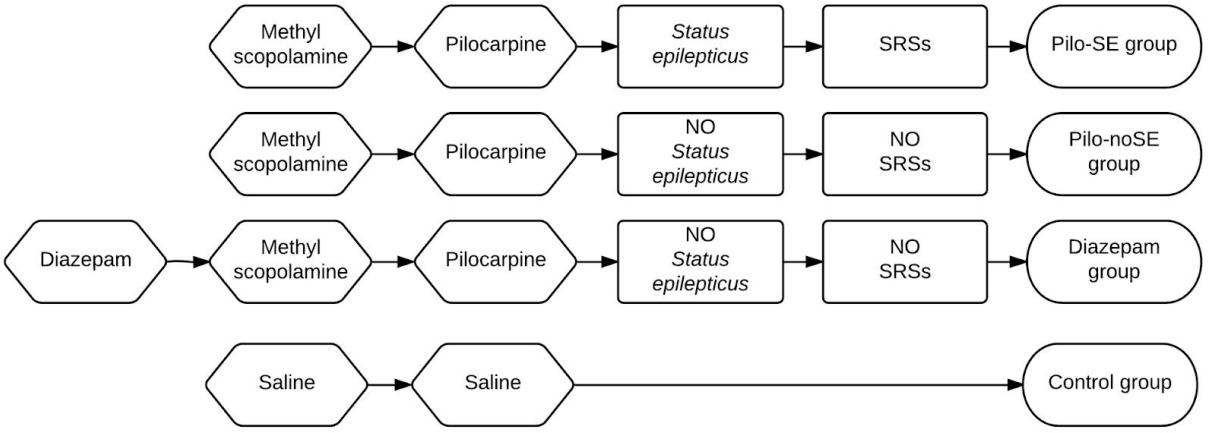

Figure 3. Protocol of the injections for establishing experimental groups.

Behavioral observation was performed for the period of 2 hours; severity of the behavioral manifestations were defined according to the modified Racine's scale (Hellier & Dudek, 2005). Stages 5 and 6 were defined as convulsive SE (Table 2). At the end of observation animals were kept 1 per cage in the controlled environment.

To evaluate the presence of SRSs animals were visually observed for 8 hours per day during the dark phase from the 7th day after the induction until at least one spontaneous seizure was detected.

Table 2. The scale of seizures severity based on behavioral manifestations.

Stages	Behavioral sings
Stage 0	no abnormality
Stage 1	mouth and facial movement, shivering
Stage 2	head nodding, stiff tail
Stage 3	forelimb clonus, chewing
Stage 4	rearing with forelimb clonus, tonic immobility
Stage 5	rearing and falling with forelimb clonus (tonic clonic-seizure)
Stage 6	running seizures and involuntary jumping in the cage

Modified from (Karlócai et al., 2011).

Afterwards, animals were divided in 4 groups:

- Pilo-SE = received methyl scopolamine (MSC) and Pilocarpine, entered to the SE and after a seizure-free period developed spontaneous recurrent seizures (SRSs)
- Pilo-noSE = received MSC and Pilocarpine, did not exhibit SE and after a seizure-free period did not develop SRSs.
- Diazepam treated = received Diazepam before MSC and Pilocarpine did not exhibit SE and after a seizure-free period did not develop SRSs.
- Control group = received a corresponding volume of saline.

Fecal sample collection

Fecal samples were obtained 2 days before and 40 days after the induction of the SE. Samples for the future inoculations were collected in a sterile 1.5 ml plastic vials prefilled with the sterile 30% Glycerol solution, frozen and stored in -80°C until use for the subsequent inoculation of recipients.

Donor material used for the analysis was collected in the in sterile 1.5 ml tubes without Glycerol solution, and immediately frozen in -80°C.

Due to the fact that animals of Pilo-SE group were hypersensitive and difficult to handle, fecal samples from all groups were collected not directly in the tubes, but by letting mice defecate normally in the sterile empty cages. Next, fecal pellets were immediately picked up with the sterile pincet and freezed as described above.

Characterization of bacterial composition in fecal biobank by cultivation of bacterial colonies

To determine a presence of bacterial forms in fecal samples, cultivation of bacteria colonies and followed matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed (Fig. 4). This type of analysis was reported to be rapid and sensitive alternative to genomic-based approaches for identification of the microorganisms (van Veen, Claas, & Kuijper, 2010).

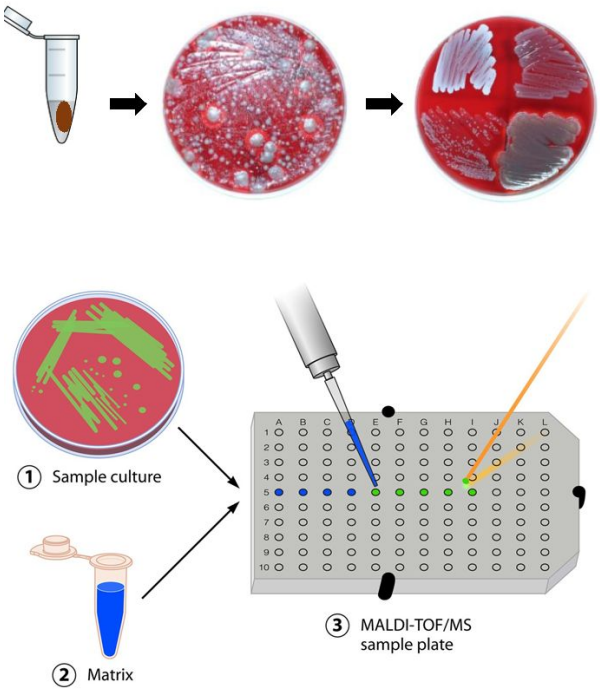

Figure 4. Schematic flow-chart of the MALDI-TOF MS analysis

<u>Cultivation</u>

Frozen fecal samples were first weighed, dissolved in 2 ml of a sterile physiological solution and than mixed for 30 minutes at the room temperature. Afterwards, the aliquote (0.5 ml) of suspension was used for a cultivation in a Petrie's dish with a blood agar substance. Moreover, using the "umbrella" method (i.e. dissolving of primary suspension by adding a sterile physiological

solution) were prepared 10, 100 and 1000 times “diluted” samples. This step was necessary to examine presence of specific bacteria population in samples by elimination of interfering between fast and slow growing bacterial cultures. Aliquots (0.5 ml) of every diluted primary suspension were as well cultivated. In total, 5 technical repetitions of every prepared suspension were used for cultivation on a blood agar under aerobic condition for 18 hours at 37° C. Grown colonies of bacteria were analysed by MALDI-TOF mass spectrometry as described elsewhere (Kolínská, Spanělová, Dřevínek, Hrabák, & Zemličková, 2015).

MALDI-TOF MS

Small amount of individual bacterial colonies was deposited in ten replicates of each sample onto a polished steel MALDI plate in the form of a thin film covered with 1 µl of matrix solution, comprised of a saturated solution of α-cyano-4-hydroxycinnamic acid (Bruker) in 50% acetonitrile (Sigma-Aldrich) and 2.5% trifluoroacetic acid (VWR) and allowed to dry. Mass spectra were acquired using a MALDI-TOF mass spectrometer (Microflex LT; Bruker Daltonics, Bremen, Germany).

Microflex was equipped with a nitrogen UV laser (λ =337 nm) at a frequency of 20 Hz with pulsed ion extraction and automated spectra acquisition. Prior to the measurement, the instrument was calibrated with Bruker Bacterial Test Standard Escherichia coli (# 25534) using multipoint quadratic calibration curve. A total of 40 laser shots were accumulated from each sample. The ions were accelerated into the mass analyzer using 20.00-kV acceleration and 18.65-kV extraction voltages. The mass range of spectra analyzed was from 2 000 to 20 000 m/z. Mass spectra were processed using the software Flex Analysis (version 3.4; Bruker Daltonics).

Following taxonomy database were used to determine individual bacteria species:

1. Bruker Taxonomy 2017 – 7311.
2. Security Relevant Library for Highly Pathogenic Microorganisms.
3. Database of new *Acinetobacters* (Radolfova-Krizova, Maixnerova, & Nemec, 2016).
4. New taxons of *Staphylococcus,* NRL/St (Švec et al., 2015).

Below mentioned individual bacteria species were determined:

- *Acinetobacter johnosnii* is a Gram-negative, strictly aerobic bacteria usually inhabiting environment and occasionally colonizing human skin (Montaña et al., 2016). It can rarely cause human infections, especially those of CNS. Several case reports were published regarding *Acinetobacter johnosnii* as a causative pathogen of the meningitis (Chang, Lu, Huang, & Chuang, 2000).
- *Bacillus cereus* is a widely-distributed, gram-positive or variable. B. cereus rarely causes bacteremia, mainly in immunocompromised individuals (Brouland, Sala, Tusgul, Rebecchini, & Kovari, 2018).
- *Enterococcus faecalis* - Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. It can be a cause life-threatening infections in humans, and it was shown to have high levels of antibiotic resistance.
- *Escherichia coli* - Gram-negative, facultative anaerobic bacterium. E. coli can secret pro-inflammatory neurotoxins that were recently were shown to influence the function of neurons in the CNS. It was reported that in brain lysates from the patients with Alzheimer's disease the presence of bacterial lipopolysaccharide (LPS) was determined recognising

microbiota-derived LPS contribution to the inflammatory processes in the CNS (Zhao et al., 2017).

- *Lactobacillus murinus* is a Gram-positive, facultative anaerobic or microaerophilic bacteria that makes major part of the lactic acid bacteria group - one of the most numerous presented in different body sites (GIT, urinary tract, genital system). In animal model abundant presence of the *Lactobacillus spp.* were reported to correlate with the low level of inflammation (A. K. Hansen, Friis Hansen, Krych, & Nielsen, 2014) and to be able to modulate intestinal dendritic cell functions (Huang, Shen, Liang, & Jan, 2016).
- *Lysinibacillus sphaericus* (reclassified - previously known as *Bacillus sphaericus*) (Ahmed, Yokota, Yamazoe, & Fujiwara, 2007) is a Gram-positive bacterium commonly distributed in soil.
- *Staphylococcus spp.* is a genus of Gram-positive bacteria that, compounding Firmicutes phylum, is frequently distributed in the nose, respiratory tract, and on the skin. CNS infections caused by *Staphylococcus spp.,* are uncommon and may occur as a complication of surgical procedures (eg. postoperative meningitis) or hematogenous dissemination from distant bacterial reservoir (Aguilar et al., 2010).

Characterisation of the microbiota composition in fecal biobank by 16S ribosomal RNA (rRNA) gene sequencing

DNA isolation

Fecal pellets from mice were collected in sterile microtubes and stored at –80°C until bacterial DNA was extracted. DNA of the fecal samples was isolated with DNeasy PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions with the slight modification: the temperature during primal vortexing was increased till 65°C. The quality and quantity of DNA were assessed (NanoDrop 1000, Thermo Scienti c, Waltham, MA).

High-throughput sequencing

Tag-encoded 16S rRNA high-throughput sequencing (NextSeq, Illumina) was used to determine the composition of the fecal microbiota. The V3 region (~ 190 bp) of the 16S rRNA gene was amplified using primers compatible with Nextera Index Kit (Illumina): NXt_388_F: 5′ -TCGTCGGCAG CGTCAGATGT GTATAAGAGA CAGACWCCTA CGGGWGGCAG CAG-3′ and NXt_518_R: 5′ -GTCTCGTGGGC TCGGAGATGTG TATAAGAGAC AGATTACCGC GGCTGCTGG-3′ (Integrated DNA Technologies). PCR reactions were performed on on a SureCycler 8800 using mixture of 12 µL AccuPrime™ SuperMix II (Life Technologies), 0.5 µL of each primer (10 µM), 5 µL of genomic DNA (~ 20 ng/µL) and 7 µ of nuclease-free water (total volume of 25 µL). PCR conditions were applied as described below: 95 °C for 2 min; 33 cycles of 95 °C for 15 s, 55 °C for 15 s and 68 °C for 30 s; followed by final step at 68 °C for 5 min. To incorporate primers with adapters and indexes, PCR reactions contained 12 µL Phusion High-Fidelity PCR Master Mix (Thermo Fisher

Scientific), 2 µL corresponding P5 and P7 primer (Nextera Index Kit), 2 µL PCR product and nuclease-free water for a total volume of 25 µL. Cycling conditions: 98 °C for 1 min; 12 cycles of 98 °C for 10 s, 55 °C for 20 s and 72 °C for 20 s; and 72 °C for 5 min. Purification of amplified fragments with adapters and tags were performed by using AMPure XP beads (Beckman Coulter Genomic). Prior to library pooling clean constructs were quantified using a Qubit Fluorometer (Invitrogen) and mixed in approximately equal concentrations to ensure even representation of reads per sample followed by NextSeq (Illumina) sequencing performed according to the instructions of the manufacturer.

First step after receiving the raw data set containing pair-ended reads with corresponding quality scores was to merge and trim it using fastq_mergepairs and fastq_filter scripts implemented in the UPARSE pipeline (Edgar, 2013). The minimum overlap length of trimmed reads (150 bp) was set to 100 bp. The minimum length of merged reads was 150 bp. The max expected error E = 2.0, and first truncating position with quality score $N \leq 4$. Purging the dataset from chimeric reads and constructing de novo Operational Taxonomic Units (OTU) were conducted using the UPARSE pipeline (Edgar, 2013). The green genes (13.8) 16S rRNA gene collection was used as a reference database (McDonald et al., 2012). Quantitative Insight Into Microbial Ecology (QIIME) open source software package (1.8.0 and 1.9.0) was used for subsequent analysis steps (Caporaso et al., 2010).

The α diversity measures expressed for an observed species (sequence similarity, 97% OTU) value were computed for rarefied OTU tables (48000 reads per sample) using the α rarefaction workflow (QIMME v1.8.0). Differences in α diversity were determined by using a t test-based approach according to the nonparametric (Monte Carlo) method (999 permutations)

implemented in the Compare α Diversity workflow (QIMME v1.8.0)

Principal coordinate analysis (PCoA) was conducted with the jackknifed beta diversity workflow based on 10 distance metrics calculated using 10 subsampled OTU tables. The number of sequences taken for each jackknifed subset was set to ~ 85% of the sequence number within the most indigent sample (48000 reads/sample). Adonis, a nonparametric statistical method was used to evaluate group differences using weighted and unweighted UniFrac (Lozupone & Knight, 2005) distance matrices that were generated based on rarefied (48000 reads/sample) OTU tables. The relative distribution of the genera registered was calculated for unified and summarized in the genus level OTU tables.

The database output for genus *Candidatus Arthromitus* was reflected in the 'Results' and 'Discussion' sections.

Recently it was reported that the bacterium *Candidatus Arthromitus* truly inhabits arthropod guts and belongs to *Lachnospiraceae (Thompson, Vier, Mikaelyan, Wienemann, & Brune, 2012)*. To avoid the mismatch of two appeared to be distinct lineages the provisional name '*Candidatus Savagella*' was proposed. Last one belongs to the family of *Clostridiaceae* and refers to the vertebrate Segmented Filamentous Bacteria (SFB) that was observed in the present study.

Results

Induction of the epilepsy

From a total of 40 animals injected with MSC+Pilo:

- ➢ 14 entered to the SE (Pilo-SE group);
- ➢ 11 entered to the SE and consequently died;
- ➢ 15 did not enter to the SE (Pilo-noSE group).

From 18 mice pre-treated with Diazepam (Diaz group) and from 12 Control no one entered to the SE.

SRSs (behavioral manifestations) were detected in all animals from Pilo-SE group. Mice pre-treated with Diazepam and Pilo-noSE group did not enter to the convulsive SE after Pilo injection and did not develop SRSs (based on behavioral observations).

Evaluation of the alterations in the brain in established experimental groups revealed that on the structural level Pilo-SE group (the only group of animals who experienced convulsive SE) differs from all the other groups (Pilo-noSE, Diazepam and Control) that appear to have similar conditions.

What is more, after the implantation of the electrodes and measuring electrophysiological activity, obtained data suggested that of the functional level, Pilo-SE, Pilo-noSE and Diazepam groups show similar alterations. Detailed evaluation will be presented in the second part of the thesis.

MALDI-TOF MS determination of bacteria populations in experimental samples

Cultivation of bacteria colonies and followed MALDI-TOF mass spectrometry analysis revealed that *Bacillus cereus, Escherichia coli*, *Lactobacillus murinus* and *Enterococcus faecalis* were equally observed in all samples in all 4 groups (Fig. 5).

Significantly less appearance of *Staphylococcus sciuri* ana *Staphylococcus aureus* were found in all groups injected with pilocarpine (Pilo-SE, Pilo-noSE, Diazepam) in comparison with Control. In addition, *Acinetobacter johnosnii*, *Staphylococcus cohnii* and *Staphylococcus xylosus* appeared only in these groups injected with the pilocarpine (Pilo-SE, Pilo-noSe, Diazepam).

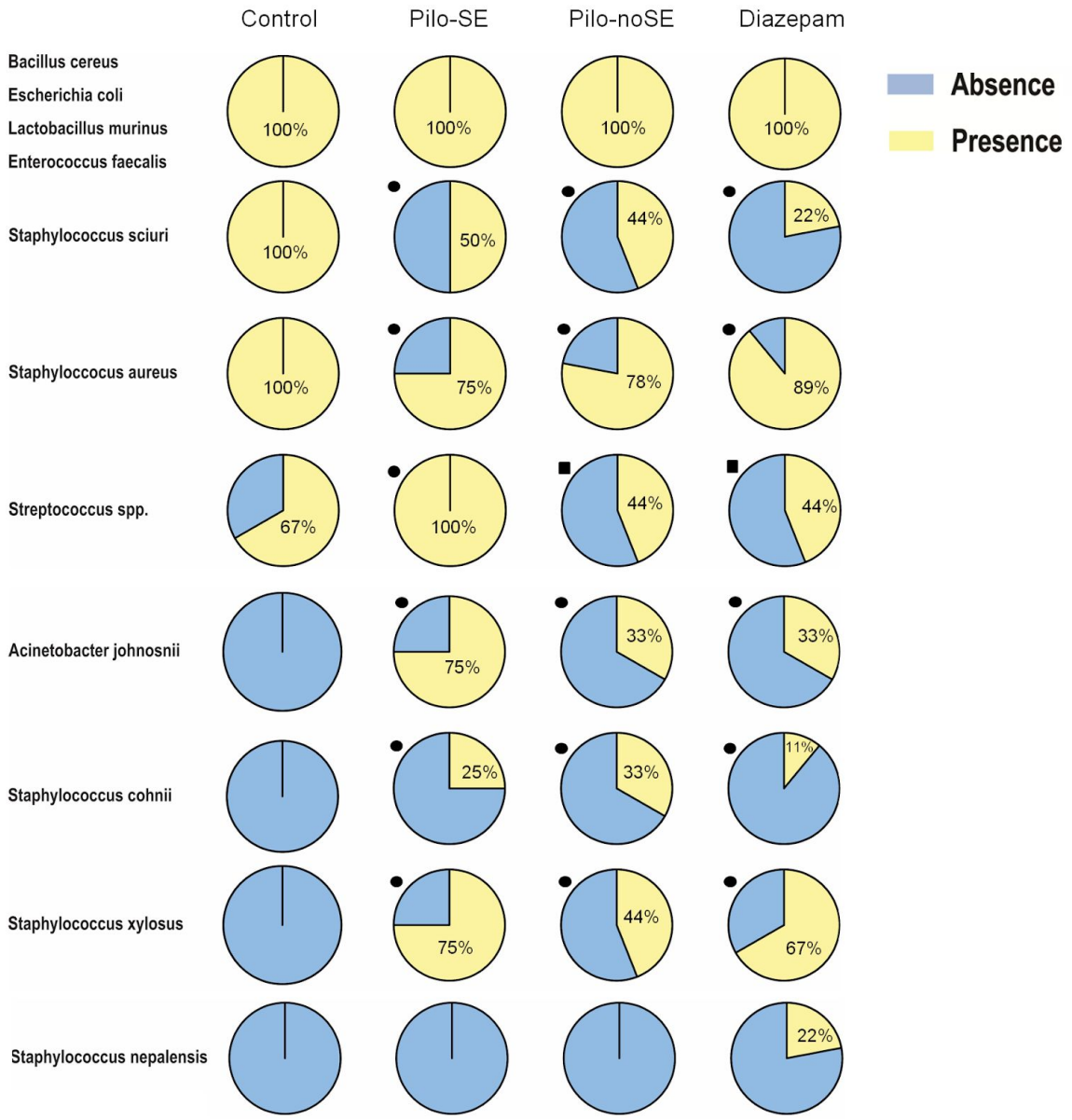

Figure 5. Appearance of isolated bacteria determined by the MALDI-TOF MS.
Control (n=3); Pilo-SE (n = 4); Pilo-noSE (n = 9); Diazepam (n = 9).
Two-sided chi-square test was used to evaluate the difference between the groups as described elsewhere (Ludbrook, 2008). P values <0.05 were taken as significant.
Yellow background indicates the presence of the corresponding bacteria in the analysed samples, blue background - absence. Black dot (●) indicates significant difference vs Control, black square (■) - significant difference vs Control & Pilo-SE.

Phylogenetic analysis of the microbiota composition in fecal biobank

Fecal samples from animals were analysed at two time-points:

- after habituation and before the injections (Baseline);
- 40 days after the SRSs detection (Pilo-SE group) or 40 days after the injections (Pilo-noSE, Diazepam and Control groups) in case of no SE and SRSs.

In the group of animals injected with pilocarpine, who entered to SE and developed SRSs (animals with chronic epilepsy, Pilo-SE group) there is no significant difference in number of genera (OTUs), but there is a significant difference (P = 0.028) in richness of microbiota in samples after injection (Fig. 6).

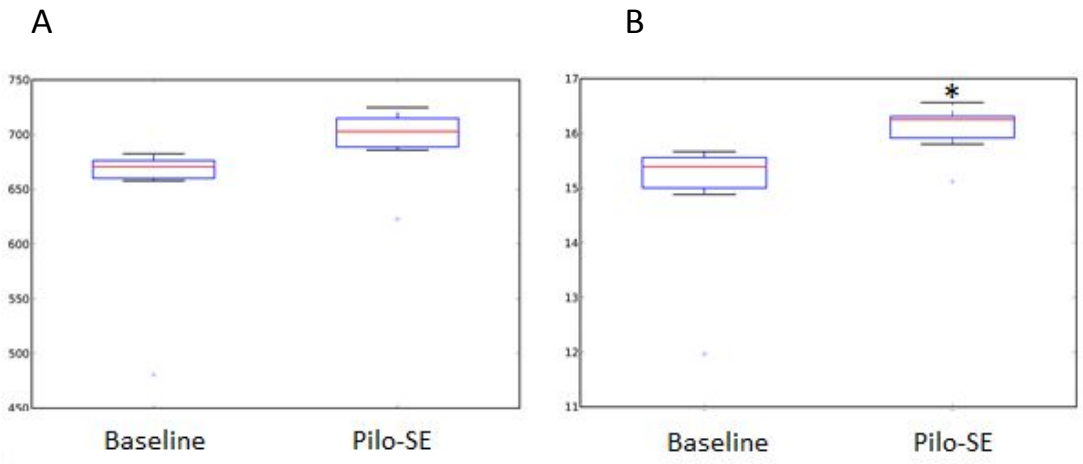

Figure 6. A. Number of observed species - operational taxonomic units (OTUs) in Pilo-SE group. B. Phylogenetic diversity between the observed bacteria in Pilo-SE group.
1.Baseline (n=6) represents group of the animals before the injection with the pilocarpine.
2.Pilo-SE (n=6) - same group of animals 40 days after the injection with pilocarpine with confirmed SRSs.

The following changes in operational taxonomic units (OTUs) in Pilo-SE group were observed (Table 3).

Table 3. Changes in OTUs in Pilo-SE group

Operational taxonomic unit	Observed changes	Baseline n=6 (Mean)	Pilo-SE n=6 (Mean)	P value
f__Alcaligenaceae; g__Sutterella	↑	0.0007	0.0976	0.0033
f__Planococcaceae;	↑	0.0000	0.0233	0.0073
f__Staphylococcaceae; g__Staphylococcus	↑	0.0000	0.0299	0.0073
f__Clostridiaceae; g__Clostridium	↓	0.0719	0.0069	0.0123
f__Prevotellaceae; g__Prevotella	↑	0.2830	1.2733	0.0163
f__Lachnospiraceae; g__Dorea	↑	0.0656	0.2156	0.0301
f__Clostridiaceae; g__Candidatus Arthromitus	↑	0.0403	0.2118	0.0370
f__Erysipelotrichaceae; g__Coprobacillus	↑	0.0111	0.1281	0.0463

Observed changes in OTUs in Pilo-SE group (40 days after the confirmed SRSs) corresponding to the baseline (before the injection of the pilocarpine).

There was no significant difference in number of genera and richness of microbiota in samples of Pilo-noSE, Diazepam and control. But the following changes in the low abundant genera were observed (Tables 4-6).

Table 4. Changes in OTUs in Pilo-noSE group

OTU	Observed changes	Baseline n=6 (Mean)	Pilo-noSE n=6 (Mean)	P value
f__Clostridiaceae g__Candidatus Arthromitus	↑	0.0438	0.3035	0.0019
f__Lachnospiraceae g__Butyrivibrio	↑	0.0052	0.1024	0.0155
f__Bacteroidaceae g__Bacteroides	↓	0.0423	0.0210	0.0528
p__Cyanobacteria c__4C0d-2 o__YS2	↓	0.1086	0.0420	0.0201
f__Lachnospiraceae g__Anaerostipes	↓	0.0234	0.0063	0.0312

Observed changes in OTUs in Pilo-noSE group (40 days after the injections: MSC, pilocarpine) corresponding to the baseline (before the injections).

Table 5. Changes in OTUs in Diazepam group

OTU	Observed changes	Baseline n=4 (Mean)	Diazepam n=4 (Mean)	P value
f__Staphylococcaceae g__Staphylococcus	↑	0.0006	0.4958	0.0038
f__Planococcaceae	↑	0.0006	0.3153	0.0051
f__Clostridiaceae g__Candidatus Arthromitus	↑	0.0294	0.4243	0.0112
p__Cyanobacteria c__4C0d-2 o__YS2	↓	0.2327	0.0472	0.0280
f__Lachnospiraceae; g__Anaerostipes	↓	0.0515	0.000	0.0369

Observed changes in OTUs in Diazepam group (40 days after the injections: Diazepam, MSC, pilocarpine) corresponding to the baseline (before the injections).

Table 6. Changes in OTUs in Control group

OTU	Observed changes	Baseline n=4 (Mean)	Control n=4 (Mean)	P value
f__Planococcaceae g__Sporosarcina	↑	0.0000	0.0135	0.0038
f__Staphylococcaceae g__Jeotgalicoccus s__psychrophilus	↑	0.0000	0.0234	0.0038
f__Staphylococcaceae g__Staphylococcus	↑	0.0000	0.0208	0.0039
c__Bacilli;o__Bacillales f__Planococcaceae	↑	0.0000	0.0120	0.0177
f__Corynebacteriaceae g__Corynebacterium	↑	0.0000	0.0281	0.0182
f__Staphylococcaceae g__Staphylococcus s__sciuri	↑	0.0000	0.0130	0.0182
c__Bacilli o__Lactobacillales f__Aerococcaceae	↑	0.0000	0.0078	0.0182
f__Clostridiaceae g__Clostridium	↓	0.0264	0.0031	0.0187
f__Lachnospiraceae g__Anaerostipes	↓	0.0191	0.0000	0.0225

Observed changes in OTUs in Control group (40 days after the injections with saline) corresponding to the baseline (before the injections).

Discussion

Experimental groups were established and fecal samples with the microbiota of interest were prepared for the future inoculations and analysis.

Cultivation of bacteria and MALDI-TOF MS analysis revealed particular species that are less present in all groups injected with pilocarpine (Pilo-SE, Pilo-noSE, Diazepam). These are *Staphylococcus sciuri* and *Staphylococcus aureus*. On the other hand, *Acinetobacter johnosnii*, *Staphylococcus cohnii* and *Staphylococcus xylosus* appeared only in these groups.

Particular species were found only in Diazepam group, but it is important to note that *Acinetobacter lwoffii* is similar to *Acinetobacter johnsohnii*, and it was detected in 11% what corresponds to one biological sample. Therefore there is a high probability of false detection or a contamination from environment.

For the same reason it could be suggested the contamination from environment in case of *Paenibacillus glucanolyticus* and *Enterobacter cloaceae*. *Staphylococcus nepalensis* and *Staphylococcus xylosus* in analyzed samples looks similar hence it can be assumed the only presence of the *S. xylosus*.

Although cultivation of bacteria already elucidated some patterns of the bacterial species distribution, it was reported that from the entire bacterial population of the GI tract of a 10^4 organisms only 10-50% can be cultivated. Thus, cultivation and following MALDI-TOF MS analysis can provide an additional insight on the presence of exact bacterial species, but it is not representative enough to characterise microbiota composition in the sample.

Recent advances in DNA-based approaches, including qPCR and high-throughput sequencing, allow to determine almost 100% of species in the sample (Zoetendal, Collier, Koike, Mackie, & Gaskins, 2004).

Thus, to reveal particular characteristics for each experimental group the DNA-based characterisation of the microbiota of the donors was performed.

Analysis of the fecal samples from the mice with TLE (Pilo-SE group) revealed that after the induction of epilepsy there is no significant difference in number of genera (P = 0.146), but there is a significant difference in richness of microbiota in samples (P = 0.028) in comparison with baseline (before the injections).

The changes in

- *f__Planococcaceae;*
- *f__Staphylococcaceae, g__Staphylococcus;*
- *f__Clostridiaceae, g__Clostridium*

correspond to the patterns in the control group and might be considered as age- and environment-related changes.

Instead, changes in

- *f__Alcaligenaceae, g__Sutterella;*
- *f__Prevotellaceae, g__Prevotella;*
- *f__Lachnospiraceae, g__Dorea;*
- *f__Erysipelotrichaceae, g__Coprobacillus*
- *f__Clostridiaceae, g__Candidatus Arthromitus;*

might be considered as specific patterns for the group of animals that were injected with the pilocarpine, entered to the SE and developed chronic epilepsy.

Interestingly, the group of the animals that were injected with the same protocol but did not enter to the SE and did not develop SRSs (Pilo-noSE group) in case of *f__Clostridiaceae, g__Candidatus Arthromitus* shows similar changes in the abundance of this genera in correspondence to the baseline.

By now, limited amount of information regarding interaction of the certain bacterial genera with the CNS, but previously it was reported that in rats *Candidatus Arthromitus* was markedly increased in chronic variable stress-induced depression model in comparison with normal controls (Yu et al., 2017).

And, at the same time, only in Pilo-noSE group of animals

- *f__Bacteroidaceae g__Bacteroides*
- *f__Lachnospiraceae, g__Butyrivibrio*

appeared to change significantly.

Bacteroides spp. represents one of the largest genera inhabiting mammalian gut, so it is difficult to associate its abundance in this experimental group with particular pattern. In general, prevalence of this bacteria was reported to be a hallmark of "Bacteroides-driven enterotype" characterized by the increased intake of the animal fat and protein (high-fat/low-fiber), so-called "Western diet" (Wu et al., 2011). Recently this type of diet was reported to promote inflammation triggered by changes in microbiota composition (Zinöcker & Lindseth, 2018).

More interesting is the fact that one of the strains *f__Lachnospiraceae, g__Butyrivibrio - Butyrivibrio fibrisolvens MDT-1* produces butyrate - one of the most abundant SCFAs which was reported to stimulate growth of mucosa in the colon and to act as protective factor in case of colon cancer and inflammatory bowel disease (Hague, Singh, & Paraskeva, 1997). Moreover, used as probiotic, *Butyrivibrio fibrisolvens MDT-1* was shown to alleviate the symptoms of the enterocolitis in rodents (Ohkawara, Furuya, Nagashima, Asanuma, & Hino, 2006).

Apart from the antiinflammatory activity *on site,* SCFAs are already recognized as one of the communicational pathways in microbiota-gut-brain alliance (Samuel et al., 2008).

As it was mentioned before, significant shifts in specific genera were observed only in animals with epilepsy (Pilo-SE group).

Recently it was reported that increased levels of *Sutterella* genus may be involved in the pathogenesis of autism (Ding, Taur, & Walkup, 2017).

Also, in the study focused on the changes in microbiota composition during the lifespan, transgenic mouse model of the Alzheimer's disease were reported to have significant increase in *Sutterella* spp. in comparison with their wild-type littermates (Ding et al., 2017).

On the other hand, the studies on mice colonized with microbiota from the patients diagnosed with MS highlighted reduction of *Sutterella* spp. as the most perceptible MS-dependent shift (Berer et al., 2017).

Presence of *Prevotella spp.* in human is associated with the enterotype characterised by low-fat/high-fiber diet (Wu et al., 2011). Hypothetically high-fiber diet could be an excellent source of the dietary fibers that under the normal conditions are transformed by intestinal bacteria to SCFAs, which, as it was mentioned before play an important role in the gut-brain interaction.

Dorea spp. also participate in fermenting dietary fibers into SCFAs. Species of this genera were shown to be associated with insulin resistance and systemic inflammation in obese rodents (Jiao et al., 2018). Moreover, prevalence of both, *Dorea* spp. and *Coprobacillus* spp. were reported to correlate with metabolic alterations which in turn are associated with cognitive impairment and cerebral hypometabolism (Sanguinetti et al., 2018).

To investigate whether these genera *per se* might interfere with the threshold of the brain excitability and subsequent seizure activity, further investigations on bigger cohort of the animals and with more precise control of the bacterial composition are needed.

Part II. Morphological and functional characterization of experimental groups

Rationale

In rodents, systemic administration of single dose of pilocarpine leads to status epilepticus (SE) and, after a seizure-free period, to a chronic condition determined by spontaneous recurrent seizures (SRSs). However, not all animals injected with convulsant dose of pilocarpine develop convulsive SE. These animals with non convulsive SE (noSE) are often excluded from the experiment or used as control group, instead of trying to understand the neurobiological substrate responsible for this "resistance". In 2009, the long-term EEG effects induced by pilocarpine on noSE rats were demonstrated (Navarro Mora et al., 2009). This study highlighted that injected with pilocarpine rats without entering to the SE can have SRSs after a long period mimicking human pathology.

Thus, as an adequate, paralleling human epileptogenesis animal model is still under the discussion it was decided to evaluate possible features in all established groups of animals including those who did not enter to the SE after the single injection of the pilocarpine (Pilo-noSE). Therefore in this part of the long-term study, characterization of bacterial population in donor groups was performed. Moreover, this part of the study is focused on functional and structural changes in the brain in order to widespread knowledge about long-term consequences of non convulsive status epilepticus in the mouse model of status epilepticus.

Materials and Methods

Magnetic resonance imaging (MRI)

For the monitoring of brain alterations in epileptic disorders in research or clinical settings MRI technique in used (Bradley & Shey, 2000).

In the present study animals underwent the MRI imaging 5 months after the induction od SE which refers to the chronical state of disease.

Images were acquired using a Biospec Tomograph (Bruker, Karlsruhe, Germany) equipped with a 4.7 T horizontal magnet with 33 cm bore (Oxford Ltd, Oxford, UK). Mice were anesthetized by inhalation of a mixture of air and O_2 containing 0.5–1% isoflurane, and placed in prone position into a 7.2 cm i.d. transmitter birdcage coil. The signal was received through a helmet coil, actively decoupled from the transmitter coil, and placed directly on the animal's head.

After a sagittal scout image, 12 contiguous 1-mm-thick slices were acquired to cover the whole-brain using a RARE T2-weighted sequence with TR=5000 ms, TE=76 ms, FOV (field of view)=2x2cm^2, NEX (number of average)=10, matrix size=128×128 for an acquisition time of 6 minutes and 40 seconds. Contrast enhancement (CE) was evaluated acquiring GRE images (TR/TE=350/15 ms, flip angle=30°, 12 contiguous 1-mm-thick slices, FOV=2x2 cm^2,matrix size=256x192, NEX=2, acquisition time=2 minutes and 14 seconds) before and two minutes after intravenous administration of 0.3 µL of Endorem® (kindly supplied by Guerbet, Aulnay-Sous-Bois, France). Diffusion tensor images (DTI) were acquired with an EPI sequence optimized for the mouse head. Imaging parameters were set as follows: TR=3000 ms, TE=36 ms, FOV=2x2 cm^2, matrix size=128x128 and 12 contiguous 1-mm-thick slices. Diffusion images were

acquired in 12 non-collinear directions with b=750 s/mm^2 and three b0 images for an acquisition time of 36 minutes.

Surgical implantation of electrodes

At the age of eight months all animals of donors group were stereotaxically implanted with bilateral cortical (epidural) and hippocampal electrodes under isoflurane anesthesia (1.5 - 2%). Cortical silver electrodes (Safina, Czech Republic) were implanted over the left and right sensorimotor cortex (AP= + 0.5 mm, ML= ±0.5 mm). In addition, isolated electrodes (PlasticsOne, USA) were implanted bilaterally into the left and right dorsal hippocampus (AP -2.3, ML ±2.5, DV 1.5). Ground and reference silver electrodes were placed over the cerebellum. All coordinates were determined according to "The Mouse Brain in Stereotaxic Coordinates", (Paxinos and Franklin, 2001). All implanted electrodes were fixed to the skull with self-curing methacrylate resin (Duracrol® Dental, Prague). At the end of the surgery, anaesthesia was terminated and animals received analgesic drug (Rimadyl, 5 mg/kg). Mice were returned to their home cages for the postsurgical recovery for 7 days.

EEG - monitoring

Electrical activity of the brain traced by the means of electroencephalography (EEG) is a crucial biomarker in a variety of neurological disorders, especially in pathologies characterised by increased brain excitability including epilepsy. It is easy applicable to the research animal model and offers high time resolution.

The EEG registration system consist of 12-channels swivel (#AC6023, Moog, USA), 8-channels bioamplifier g.BSamp (Biosignal amplifier, gTec, USA) and 16-bit analog-to-digital converter (Power-Lab 8/35, AD Instruments, USA). The Labchart software v.8 (AD Instruments, USA) was used to record bioelectric activity.

At the beginning of EEG-monitoring, animals were connected with a custom made 6-channels cables to the EEG system. Mice were individually placed into the plexiglass cages (18x18x20 cm) with free access to food and hydrogel. In addition, all plexiglass cages were placed into individual boxes with controlled 12:12 dark/light cycle, temperature control and sound isolation (Ugo basile, USA). Free movement of the animals was guaranteed by the electrical swivel mounted to the top wall of the box.

Registration of brain electrical activity was performed with the amplification 1000x at the range +/- 5V, and hardware based filters (band-pass filter 0.5-1000 Hz). Recorded signal was digitized at the sample frequency 1kHz. In total, 48h of EEG were recorded for each animal.

Analysis

Left sensorimotor and hippocampal channels in reference to cerebellum were selected for the analysis (incidence of seizures and total power). Analysis was done offline using the LabChart v.8 (AD Instruments, USA) software and custom-made Matlab script (MathWorks, Natick, MA, USA) for every animal

individually. The analysis of total power was done for each channel continuously with a 5s-window for delta (0.5-4 Hz), theta (4.5-8 Hz), alpha (8.5-13 Hz), beta (13.5-24 Hz) and gamma bands (25-70 Hz).

In future analysis relative power for each EEG-band in the 5s-window was calculated by normalization to the corresponded total power (sum of all band powers). In addition, relative total power values was firstly averaged for every hour to evaluate possible impact of the light/dark phase on the EEG-pattern. At the end, we performed average of whole period of EEG recording (i.e. 24h) for individual animals and this value was used lated for statistical analysis.

Statistical analysis was done with GraphPrism v.7 (GraphPad Software Inc., CA, USA) and Matlab (MathWorks, USA). The one-way ANOVA test followed by multiple comparisons was used for the test of significance. The level of significance was defined as $p < 0.05$.

Auditory Event Related Potentials (ERPs)

Event-related potentials (ERPs) are very small voltage fluctuations generated in the brain in response to specific events or stimuli (Blackwood & Muir, 1990). Being a time-locked EEG activity this method offers an excellent temporal resolution and may suggest an additional insight for the understanding of the functional state of the brain including both sensory and cognitive-related processes.

It provides a continuous measure of stimulus processing at different anatomical levels and it can be divided into 2 categories. The early components reflect “sensory” respond to the given stimuli and later components represents how the subject elaborate this signal, indicating ‘cognitive’ part of the information processing (Sur & Sinha, 2009).

All components can be visualized in the waveforms P1, N1, P2, N2, P3 (see Fig.7):

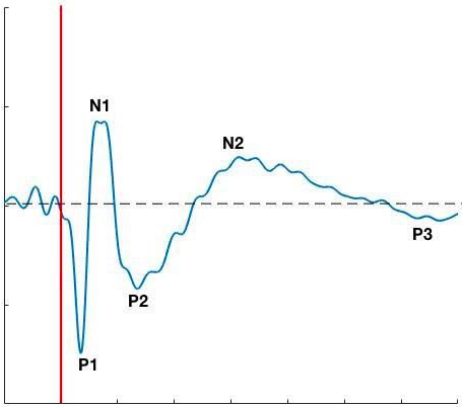

Figure 7. Schematic representation of the ERP.

- P1

The P1 peak is a positive evoked potential that in human usually appears 50 ms after the stimulus and its form can indicate a filtering process of irrelevant information (Witten et al., 2016). This peak is generated laterally in the Heschl's gyrus, in the primary auditory cortex (Liégeois-Chauvel, Musolino, Badier, Marquis, & Chauvel, 1994).

- N1

The N1 peak is a negative peak between 90 and 200 ms after the onset of stimulus which reflects the perception of the signal and detection of the changes in the auditory environment (Arnott, Bardouille, Ross, & Alain, 2011; Ullsperger, Freude, & Erdmann, 2001). It originates from: the auditory cortex, vertex and from a source in the superior temporal gyrus (Luck, 2012).

- P2

The P2 appears around 100-250 ms after the stimulus and current evidence suggests that together with the attentional allocation it is associated with a categorization process (Paiva et al., 2016).

- N2

The N2 peak was shown to be involved in categorization process, stimulus evaluation and discrimination (Vaitkevičius, Kaubrys, & Audronytė, 2015).

- P3

P3 in clinical is recognized as a positive evoked potential that appears around 300 ms and its latency has been defined as an index of cognitive efficiency (Kutas, McCarthy, & Donchin, 1977). This component was studied a lot in relation with epilepsy and P3 latency prolongation was reported to be an important clinical marker for the seizures duration and frequency (Caravaglios et al., 2001; Fukai, Motomura, Kobayashi, Asaba, & Sakai, 1990).

In the present study auditory ERP was used to evaluate functional differences between experimental groups.

For ERP registration the same set up as for the EEG was used with the sensitivity increased (voltage range +/- 1V) and sample frequency 4 kHz.

ERP was registered during the dark phase. Respond for auditory stimuli was recorded.

Based on the previous studies conducted in our laboratory the following procedure was used:

For each animal two protocols were applied, in both protocols non-target (nt) – low frequency stimuli were presented in 80%, target (t) - high frequency stimuli were presented in 20%.

Table 7. Protocol of the audial stimuli application.

	Non-target stimuli frequency, Hz	Target stimuli frequency, Hz
Protocol 1	4	6
Protocol 2	4	8

ERPs were elicited with a passive acoustic oddball paradigm. The sinusoidal sound tones were generated by a programmable online frequency signal generator. Speakers were located 18 cm above the animal. The event labels were sent by custom-made script in Matlab (R2015a, Mathworks, USA) and StimTracker (Cedrus, San Pedro, USA) via serial output into digital inputs of PowerLab and stored directly in the Labchart file.

In total in each protocol 320 stimuli were applied. Stimuli were interspersed randomly, each tone with 50 ms of duration and 78 db SPL of loudness. Interstimulus interval was also randomized from 1.7 seconds to 2.1 seconds. Two stimulation protocols were used randomly, interval between protocols was 24 hours.

Analysis of the signal

Offline filtering was performed using LabChart, Notch filter (50 Hz) was applied and the bandpass was set at 0.1-70 Hz. ERP analysis was performed using a custom-made script in Matlab (R2016b, Mathworks, USA).

Firstly, the zero-phase filtering and artefacts removing were done. Then, segmentation of the EEG signal was performed: in the window -600 before and 1100 ms after the trigger (stimuli). From all corresponding segments for t and nt stimuli (for every channel, every mouse) , the grand average ERP was constructed and used for the later analysis.

This grand average ERP was visualized for every single subject and after the visual inspection of the curves some animals were excluded due to the bad quality of the signal.

The amplitude and latency for P1, N1, P2, N2 and P3 peaks were calculated.

Results

MRI

Neuroimaging was performed 5 month after the induction of status epilepticus to evaluate structural alterations of brain regions of microbiota donors. Diffusion Weighted Images (DWI), T2-weighted images and perfusion maps were obtained and following analysis were done for auditory cortex, cerebellum, entorhinal cortex, hippocampus, sensorimotor cortex, thalamus and visual cortex. Analysis of the DWI and T2-weighted images did not show significant alterations between the experimental groups. Similarly, evaluation of the contrast enhancement did not reveal alteration of perfusion in all brain regions of interest except hippocampus.

In hippocampus, the one-way ANOVA analysis with following Holm-Sidak post hoc test ($F(3, 37) = 4.703$, $P=0.007$) showed significant differences in contrast enhancement Pilo-SE versus Diazepam and Pilo-noSE groups (Fig 8A).

Similarly, analysis of the axial diffusivity showed significantly increased coefficient (one-way ANOVA with following Holm-Sidak post hoc test: $F(3, 41) = 12.33$, $P < 0.0001$) in the Pilo-SE group in comparison with other experimental groups (Fig 8B). Likewise, the ratio of normalized signal of radial diffusivity was significantly higher (one-way ANOVA with following Holm-Sidak post hoc test ($F(3, 41) = 9.401$, $P < 0.0001$) in the Pilo-SE group in comparison with other groups (Fig 8C).

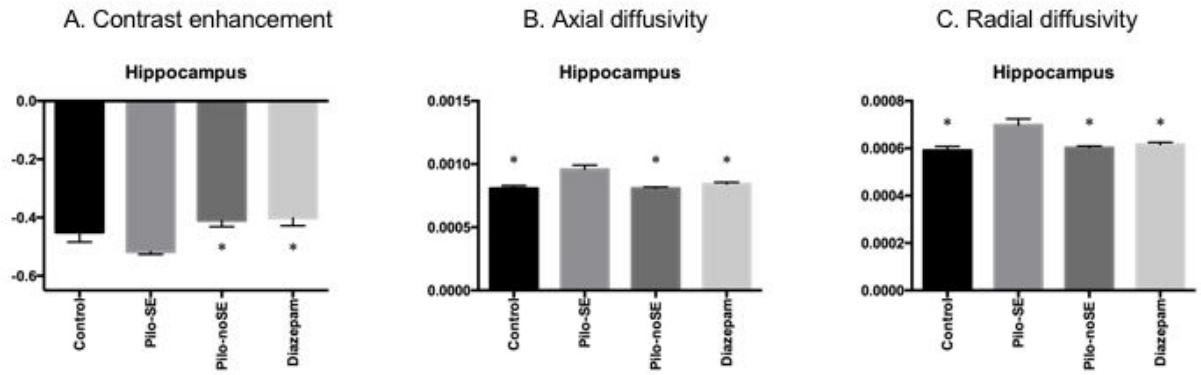

Figure 8. MRI imaging of the animals 5 months after the induction of the SE (Control n=6, Pilo-SE n=11 , Pilo-noSE n=13, Diazepam n=15). Asterisk indicates the significant ($p<0.05$) difference in comparison with Pilo-SE group. A. The coefficient of the contrast enhancement ratio in donors of microbiota. B. The normalized signal of axial diffusivity C. The normalized signal of radial diffusivity.

EEG: occurrence of seizures and power spectral density analysis

In a long-term observation (8 months after drugs injection) in all groups, except controls, epileptic seizures were found. Epileptic activity were represented by slow polymorphic spikes (2.5-4.5 Hz) and fast-runs activity (6-14 Hz). Whereas in Pilo-noSE and Diazepam groups mostly ictal hippocampal seizures were present (~89 %), in the Pilo-SE mice ~76% of seizures were present in hippocampus and sensorimotor cortex as a result of secondary generalization. Moreover, in all animals injected with pilocarpine, a continual interictal activity represented by short discharges (~3 s) and sharp spikes was observed in hippocampal and cortical EEG. Also in control group isolated spikes and slow polymorphic discharges were observed, however this activity never been continual, therefore, it can be associated with electrodes implantation and also with aging.

In analysis of seizures we focused on presence and duration of fast-runs activity (6-14 Hz) in the hippocampal channel with duration $\geq$ 5s. One-way

ANOVA analysis and following Sidak post hoc test revealed significant differences in the incidence of seizures (Fig. 9) between Pilo-SE and Pilo-noSE groups (F (2, 11) = 5.4, P = 0.0238). In groups after Pilo injection all other parameters were not significantly different.

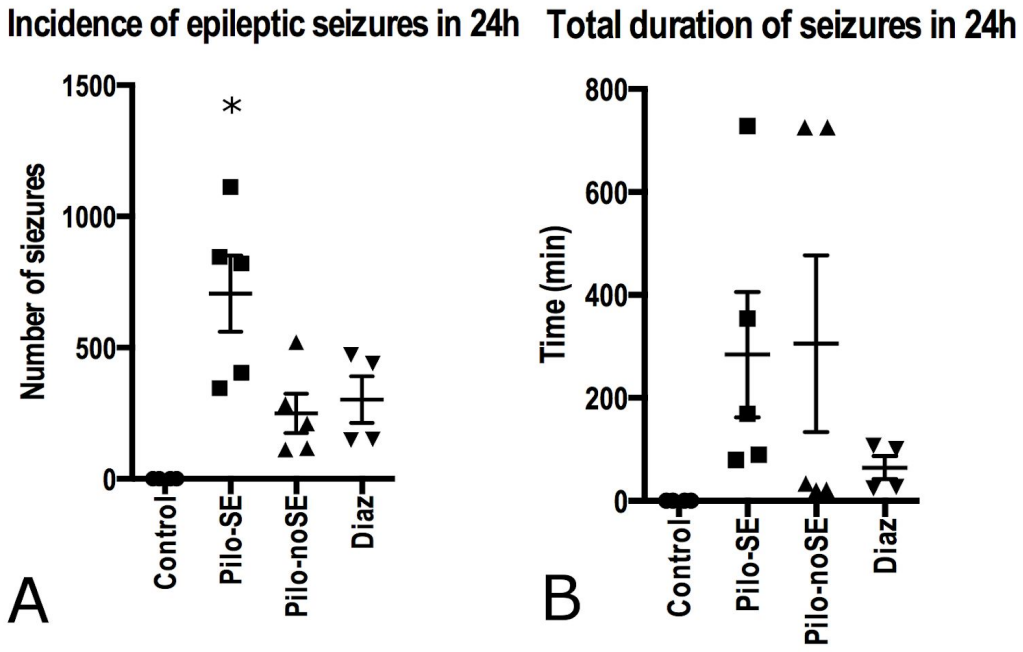

Figure 9. Representation of the:
A. incidence of the epileptic seizures in donors of the microbiota in hippocampal channel (Mean±SEM).
Axis Y represents amount of seizures counted in the 24 hours of EEG monitoring . On the axis X - experimental groups. Each symbol represent the value for individual subject.

Asterisk (∗) indicate significant difference in comparison with the Pilo-noSE group.

One-way ANOVA analysis and following Sidak post hoc test revealed significant differences between Pilo-SE and Pilo-noSE groups (F (2, 11) = 5.4, P = 0.0238).
B. Total duration of the seizures (Mean±SEM) in the 24 hours of EEG recording.
Axis Y represents total time of the seizures duration (in minutes) calculated in the 24 hours of EEG monitoring . On the axis X - experimental groups.

Using bipolar derivation (hippocampus channel to sensorimotor cortex channel) amount of sharp-spikes (Fig. 10) in all groups was calculated. The statistical analysis using one-way ANOVA and following Sidak post hoc test revealed the significant significant increase of spikes count by ~ 30-45% in the Pilo-SE and Pilo-noSE groups in comparison to the Control group ($F(2, 9) = 0.4888$, $P = 0.6287$).

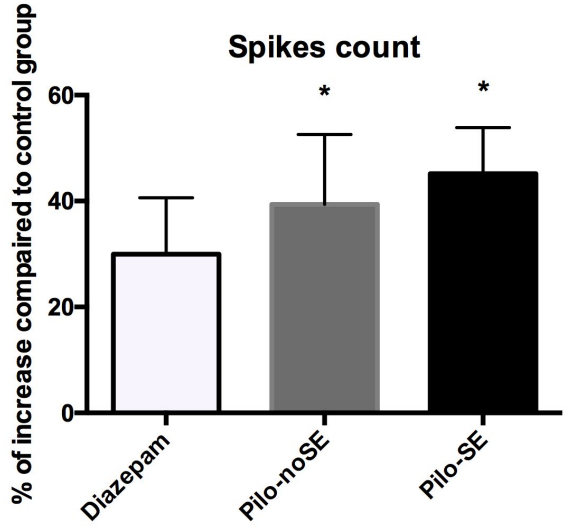

Figure 10. Appearance of sharp-spikes during EEG monitoring (24h) in microbiota donors (Mean+SEM).
Axis Y represents increased occurence of spikes in percentage in comparison to Control group. On the axis X - experimental groups 8 months after Pilo injection. Asteriks (*) indicate the significant difference in comparison with the Control group.

Moreover, the power spectral density (PSD) analysis was obtained to characterize changes in individual EEG frequencies in microbiota donors. Analysis of PSD did not showed differences in between light and dark phases of the EEG monitoring therefore PSD for all period of observation (24h) was averaged for every individual animal and used for statistical evaluation. The changes in XXX frequencies were observed (Fig . 11).

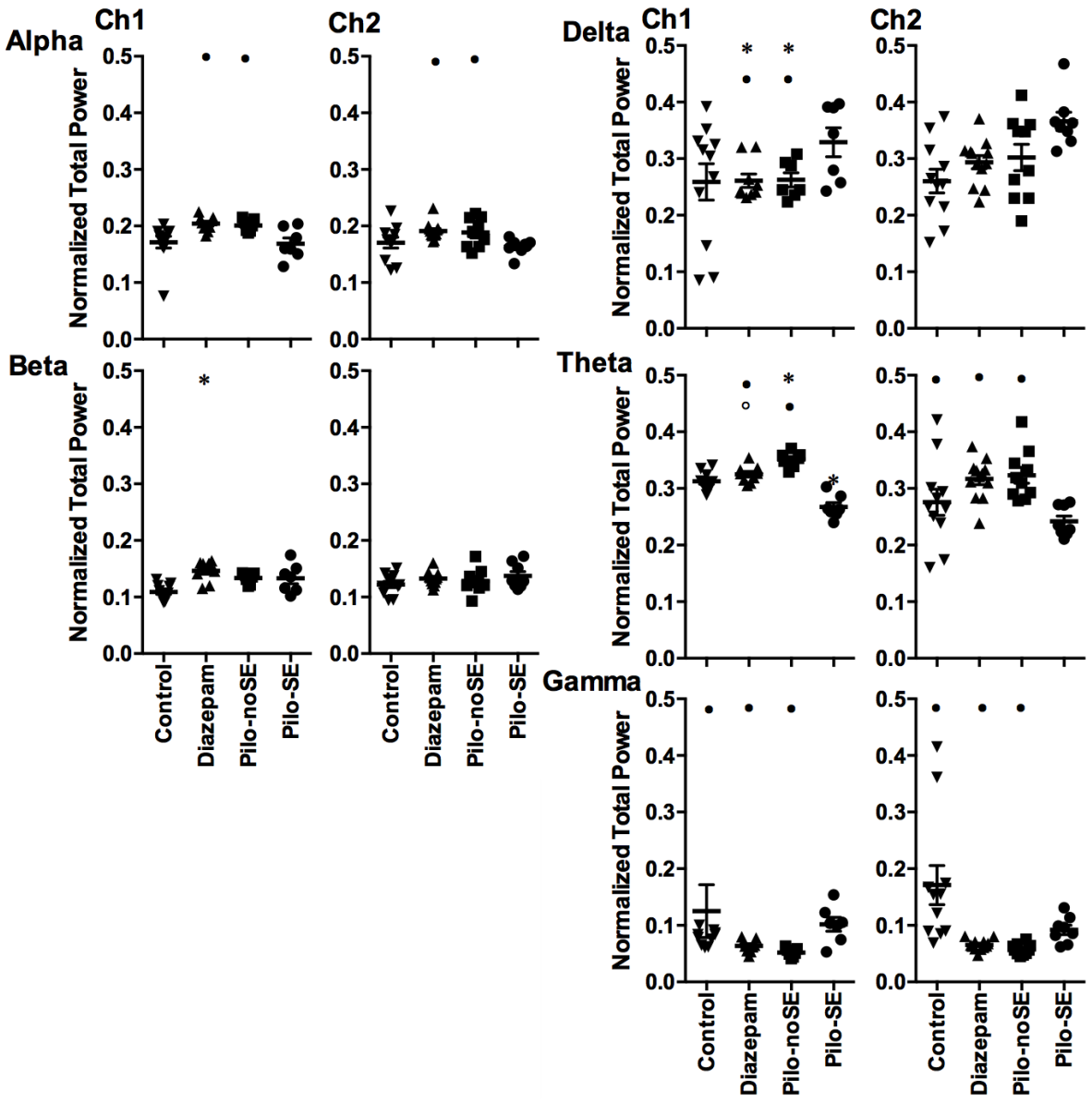

Figure 11. Representation of the normalized total power in the sensory-motor cortex (Ch1) and hippocampus (Ch2) of donors of the microbiota (Mean ± SEM).
Axis Y represents normalized total power. On the axis X - experimental groups.
Significant differences ($p < 0.05$) determined by one-way ANOVA and following Sidak post hoc test are represented with asterisks (*) indicating significant difference in comparison with the Control group, with black dots - versus Pilo-SE (•), empty circles (°)- versus Pilo-noSE .

Besides, ratio between low/high frequencies to the dominant frequency in rodent's brain (theta) was calculated (Fig. 12).

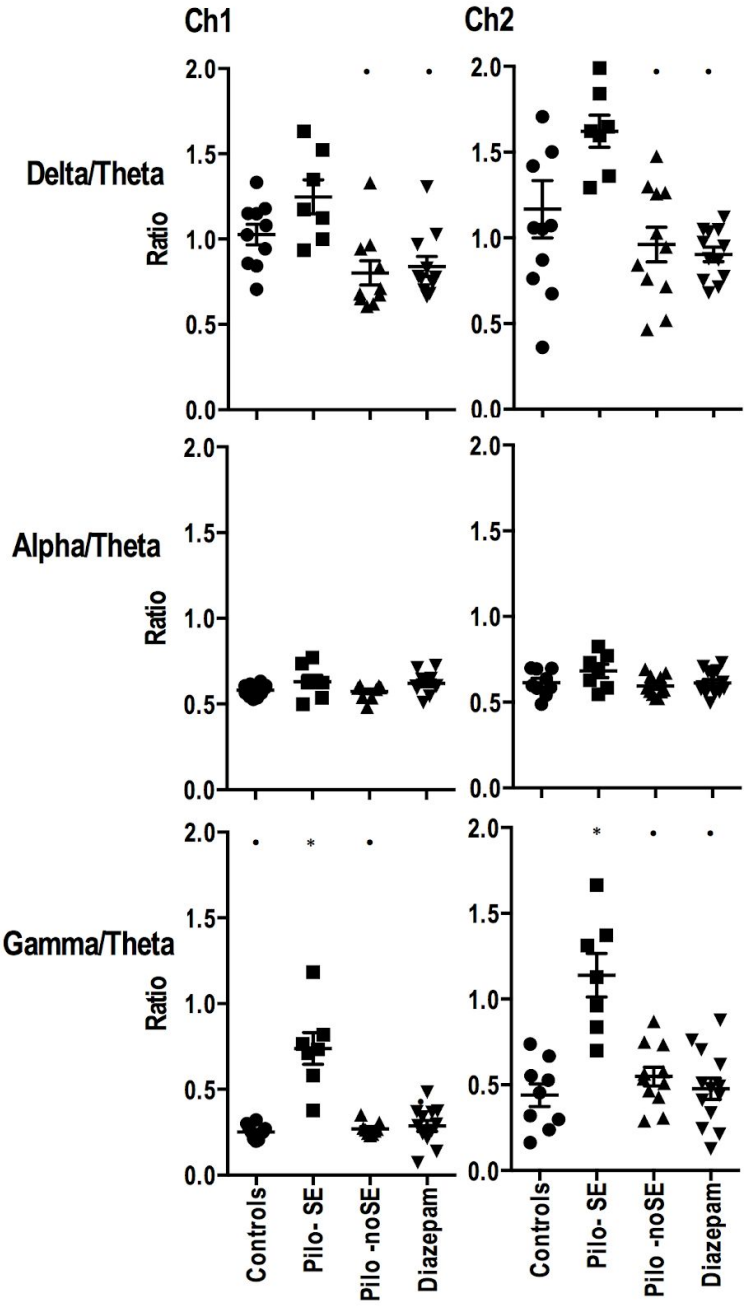

Figure 12. Representation of the ratio of the total power in the sensory-motor cortex (Ch1) and hippocampus (Ch2) of donors of the microbiota (Mean ± SEM).
Axis Y represents the ratio. On the axis X - experimental groups.
Asterisks indicate significant difference in comparison with the Control group, black dots - versus Pilo-SE.
Significant differences ($p < 0.05$) determined by one-way ANOVA and following Sidak post hoc test are represented with black dot (●) - in comparison to Pilo SE asterisk (✻) - in comparison to Control.

Auditory ERPs

As it was mentioned above, to evaluate a long-term consequences of SE on the cognitive processing, auditory ERPs were performed 8 months after drugs administration. The results are represented in Fig. 13.

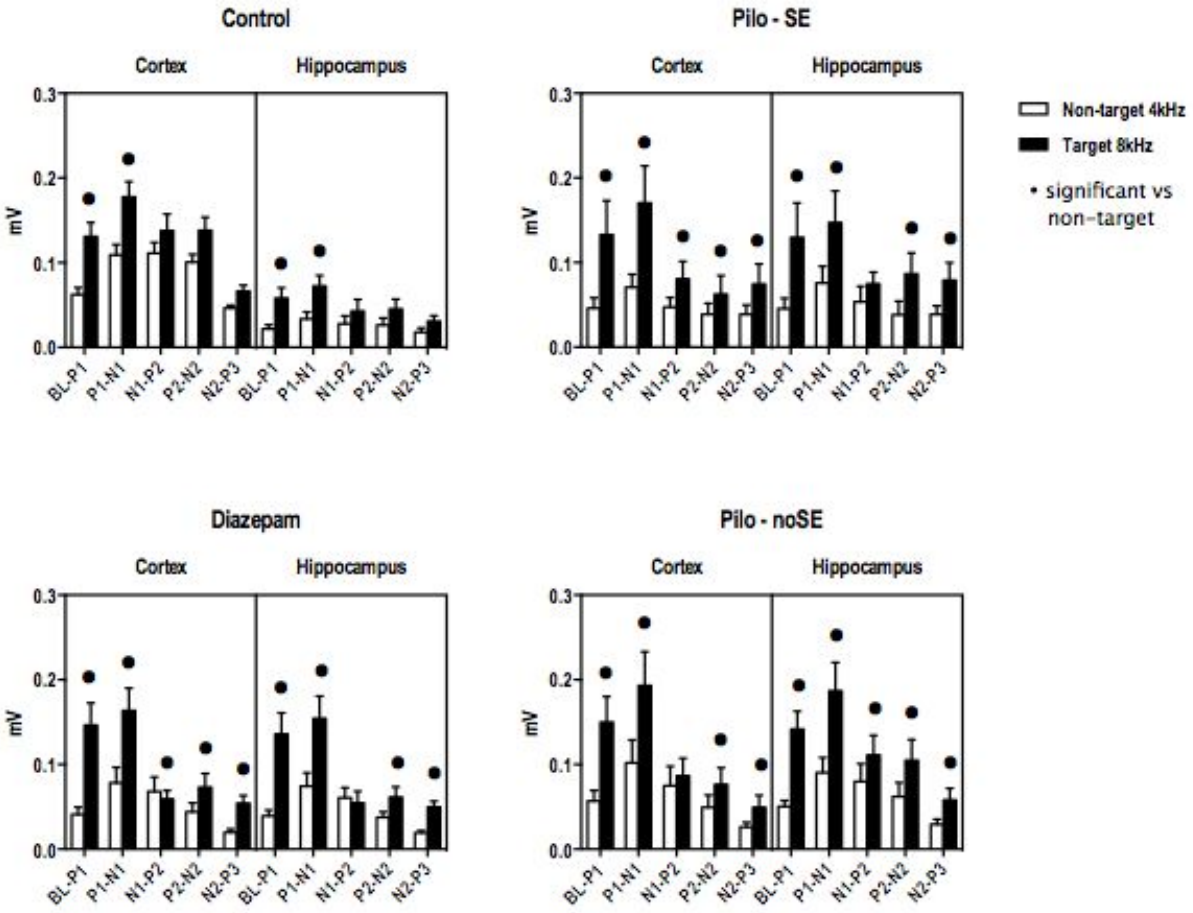

Figure 13. Amplitude of the auditory evoked potentials in the sensory-motor cortex and hippocampus of donors of the microbiota (Mean ± SEM).
White bars represent non-target stimuli (4 kHz), black bars - target (8 kHz). Axis Y represents amplitude of the evoked responses in mV. On the axis X - grouped columns corresponding to the ERP peaks.
Black dot (●) indicates significant difference in the amplitude between non-target and target stimuli.

Two protocols of auditory ERPs were used 4/6 kHz(nt/t) and 4/8 kHz (nt/t) always in 80/20 % of stimuli representation. In our study we did not found any relevant and significant changes with 4/6 kHz protocol was used (data not shown). Therefore we focused on ERP changes in the 4/8 kHz (nt/t) stimuli.

In a Control group two-way ANOVA analysis revealed significant differences in both sensorimotor cortex and dorsal hippocampus channels in between non-target and target stimuli ($F (1, 120) = 27.74$, $P < 0.0001$). Following Sidak post hoc test showed significantly ($P < 0.05$) higher amplitude of target response for BL-P1 and P1-N1.

In Pilo-SE group two-way ANOVA analysis revealed significant differences in both sensorimotor cortex and dorsal hippocampus channels in between non-target and target stimuli ($F (3, 100) = 8.701$, $P < 0.0001$). Following Sidak post hoc test showed signif ($P < 0.05$) higher amplitude of target response for BL-P1, P1-N1, N1-P2 (only in cortical channel), P2-N2, N2-P3.

In Pilo-noSE group two-way ANOVA analysis revealed significant differences in both sensorimotor cortex and dorsal hippocampus in between non-target and target stimuli ($F (3, 60) = 10.62$, $P < 0.0001$). Following Sidak post hoc test showed significant ($P < 0.05$) higher amplitude of target response for BL-P1, P1-N1, N1-P2 (only in hippocampal channel), P2-N2, N2-P3.

In Diazepam two-way ANOVA analysis revealed significant differences in both sensorimotor cortex and dorsal hippocampus channels in between non-target and target stimuli ($F (3, 160) = 15.37$, $P < 0.0001$). Following Sidak post hoc test showed significant ($P < 0.05$) higher amplitude of target response for BL-P1, P1-N1, N1-P2 (only in cortical channel), P2-N2, N2-P3.

Discussion

Despite the fact that epilepsy appears to be one of the most severe neurological disorder in the world and a great number of the clinical and laboratory *in vivo* studies are focused in revealing and elucidating the exact pathogenesis and possible treatments of this disorder, to date there are still debates on the adequate animal model that will resemble human pathology.

It was shown that SE - a crucial event in the animal model of the epileptogenesis - in humans is not so valuable for the prognosis: in patients experienced acute symptomatic SE the risk of spontaneous seizure activity after a 10 years follow-up was only 41% (Hesdorffer, Logroscino, Cascino, Annegers, & Hauser, 1998).

What is more, in the experimental model of the TLE (pilocarpine-induced epilepsy in Wistar rats) it was shown that the group of the animals that was injected with the pilocarpine but did not experienced convulsive SE, in the long-term prospective (8/+22 months after) exhibit spontaneous seizure activity disregarding the lack of the initial acute injury (SE).

The present study reports that NMRI mice who did not enter to the convulsive SE (Pilo-noSE), after a long latency period (8 months) actually experience non convulsive seizure activity in the dorsal hippocampus.

Moreover, structural characterization of the experimental groups revealed that at the time-point of 5 months after the induction of epilepsy only Pilo-SE group had structural alterations in hippocampus that can can be a manifestation of the vasogenic edema after the acute SE. Other groups did not experienced such severe response to the same amount of the Pilo.

On the other hand, evaluation of the functional shifts and electrophysiological patterns suggests that these animals (Pilo-noSE) due to the increased presence of the gamma activity, known to be associated with the neuronal pathology (Uhlhaas, Pipa, Neuenschwander, Wibral, & Singer, 2011), have altered excitability of the brain that is also represented by shifted gamma/theta ratio.

Regarding investigation of the ERPs in these animals, data from the previous studies performed in our lab on 4 months old male NMRI mice, suggests that in intact control animals respond to the target stimulus (8kHz) is significantly higher than in the mice with the neuronal pathology. However, in present study control mice show no differences in response to target (8kHz) vs non-target (4kHz) stimuli in late components (i.e P2-N2, N2-P3). As ERPs were performed when mice were 10 months old, this can be explained by age-related conditions of the animals. In addition, another protocol of the stimulation was applied using different frequencies for the stimuli: nt - 4, t-6 kHz (data not presented here) - also in this case no differences in late components in control animals were determined.

Interestingly, all groups who received pilocarpine (Pilo-SE, Pilo-noSE, Diazepam) show significantly higher respond in the late component to the target signal in comparison with the corresponding non-target one. This finding, taken together with the higher presence of pro-pathological gamma-band in eeg in this animals can be associated with the higher excitability of the brain.

This suggests that even with the lack of the initial acute SE, animals from the Pilo-noSE group experience functional alterations similar to those of the animals after the convulsive SE.

Taking together, we suggest that the absence of the acute convulsive SE and evident structural deviations, long-term “silent” period before the appearance

of the spontaneous seizure activity and altered excitation of the brain in the animals who usually do not respond “in a normal way” to the standard injection of the pilocarpine (and therefore that are excluded from the studies), can, actually, mimic human epileptogenesis in a more physiological way. Thus, further investigations, with the particular focus on this group of animals, are required.

The exact mechanisms underlying such different response of the animals to the pilocarpine still have to be investigated and revealed. Among others, also intestinal microbiota can be an important partaker in the complex background of the host that makes it more of less susceptible to the induction of the SE.
Future DNA-analysis of the fecal samples may provide additional insight on the possible correlations between the baseline microbiota composition and response to the induction of the SE with the pilocarpine.

Indeed, already in 2012 it has been proposed that the microbiota of the laboratory rodents should be included in the hygienic standardization (Bleich & Hansen, 2012). Accumulating data suggest that the the composition and the state of the commensal microbiota can profoundly affect the performance of disease models, so it is no more a question of monitoring only potentially pathogenic species, but it is time of more “holistic” approach and control of the entire microbiota composition assuring better standardization of the animal models.

Part III. Effect of the transplanted pro-pathological microbiota on the excitability of the brain

Rationale

The aim of the study is to test the hypothesis that gut microbiota from subjects affected by neural pathology can modulate in healthy subjects excitability in CNS and finally positively correlate with the level of seizure activity.

The purpose is to determine the impact of a transplanted microbiota (from animals with epilepsy) on animals (pups) in which the disease is absent, in order to clarify whether (i) the presence of the disease can change microbiota in the gut to the point of having a “pro-pathological” microbiota; (ii) if the microbiota from animals with epilepsy can *per se* provoke the excitability of the the brain appearing through the lowering the threshold of the seizures.

In this step, the fecal microbiota obtained from the experimental groups created in Part I were transplanted to the healthy subjects.

Experimental design

General description

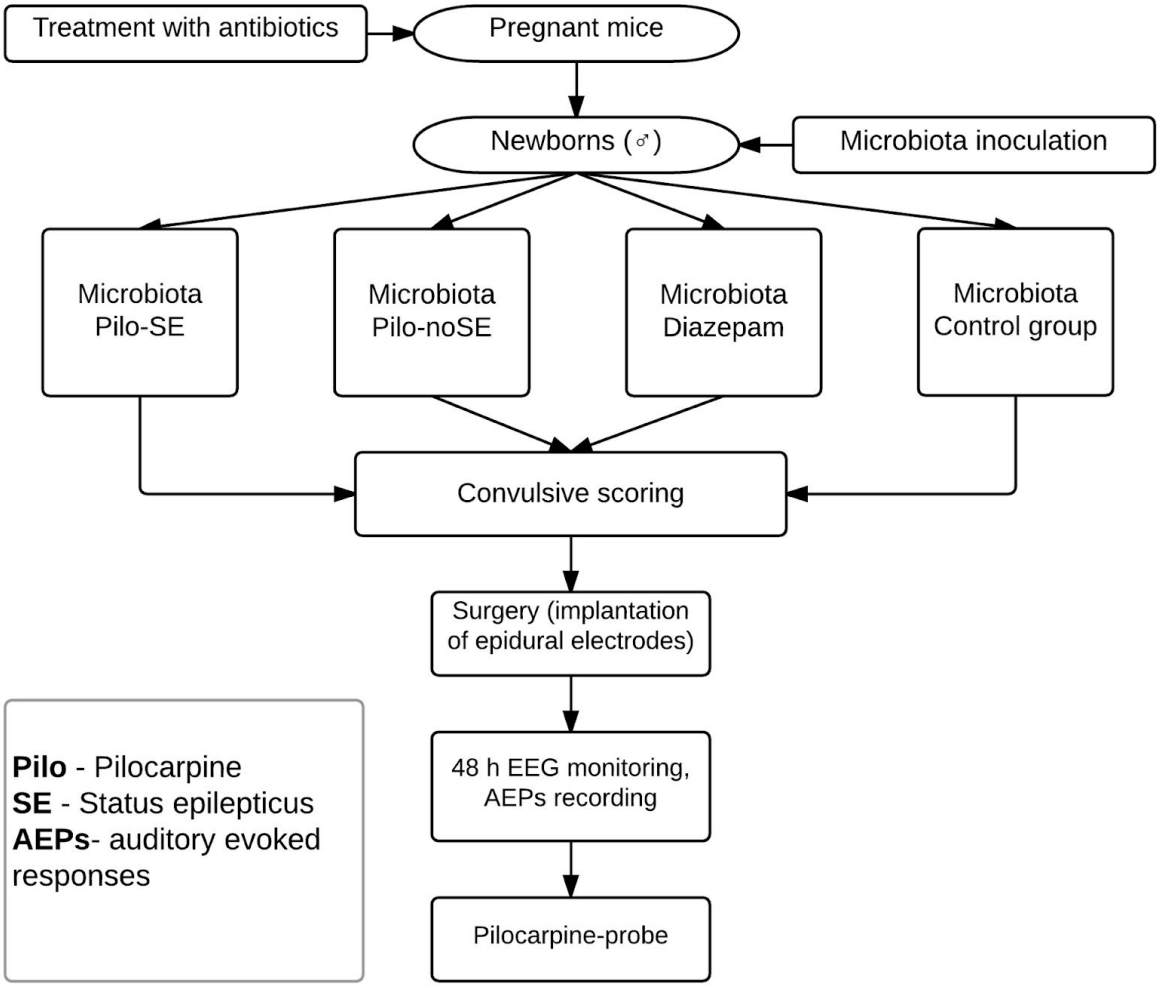

Figure 14. Flowchart of the experiment.

During the experiment animals were kept in the facility equipped with the multiple IVC cages, all manipulation were performed under the hood. This type of housing was chosen to provide optimal protection of the microbiota of interest from the environment in every step of the experiment. Lundberg et al. who tested the efficacy of the housing animals colonized with the complex microbiota in multiple IVC cages vs isolator, reported that (i) colonization was equally efficient in isolators and IVCs, (ii) the bacteria community of the donor

was exhibited in both housing systems, (iii) 16S rRNA gene sequencing did not reveal any increase in bacterial diversity in the IVC cages (Lundberg, Bahl, Licht, Toft, & Hansen, 2017).

To provide the best condition for settling down microbiota in the acceptor organism several steps before the transplantation were made:
Mice, after the habituation period of 2 weeks, were mated and from the 12th day of pregnancy till the delivery of the pups a cocktail of antibiotics was applied.
To avoid the degradation of the antibiotics in the drinking water (due to the light exposure and long-term keeping in the room temperature) and to provide optimal dosing and uninformed mixing levels, antibiotics were changed every 24 h.
Only male pups were processed as in the last step animals were went through the pilocarpine challenge.

A single oral inoculation of the microbiota of interest were showed to be the most efficient at three weeks of age (C. H. F. Hansen et al., 2012). Thus, FMT were performed twice in P21 and P24 by oral gavage and, in addition, by contaminating the cage of acceptors with fecal pellets of donors. All procedures were performed under the hood in SPF environment and during handling procedures to provide maximal microbiological safety and reduce the level of stress for the animals.

7 days after settling down the microbiota of interest mice were visually observed (4 hours per day, 3 days) to reveal the appearance of SRSs. As behavioural manifestations were not revealed, mice underwent the implantation of the cortical electrodes for the electrophysiological evaluation. For each animal 48 hours of EEG was recorded and auditory ERPs were performed.

Finally, in order to evaluate the susceptibility of the animals to epilepsy, mice underwent the pilocarpine challenge with subclinical dose (260 mg/kg, i.p.) of the drug.

Materials and Methods

Animals

NMRI mice (Charles River, France) 4 weeks old were kept in the animal facility of the University of Verona in the individually ventilated cages (IVC) with the sterile standard food and bottled autoclaved tap water *ad libitum*, without dietary variation within the study.

After the 2 weeks of habituation animals were mated. From the 12th day of pregnancy a mix of antibiotics (Ampicillin 1g/L, Vancomycin 0.5 g/L, Neomycin 1 g/L) was added to the autoclaved drinking water till the delivery of pups. The male pups were weaned to the three weeks of age, then randomized into 4 groups (Pilo-SE, Pilo-noSE, Diazepam, Control).

Gut microbiota transfer

Immediately after collecting, fecal samples from the donors were divided into aliquots and frozen at -80 in 30% Glycerol/PBS solution.

At the day of inoculation fecal samples were extracted from the Glycerol/PBS solution and vortexed with PBS.

At the age of P21 and P24 inoculated by oral gavage with corresponding fecal samples homogenized with PBS solution (50 µl/mouse) per mouse. All manipulations were performed under the hood in the time interval 9am - 11am. After the inoculation mice were kept 1 per cage in the enriched environment conditions.

Surgery

As described in Part II. With the exception that instead of the deep hippocampal electrodes cortical silver electrodes over left and right parietal

cortex (AP -2.3, ML ± 2.4) were implanted. All coordinates were calculated according to Bregma-Lambda distance for every animal according to “The Mouse Brain in Stereotaxic Coordinates”, (Paxinos and Franklin, 2001).

EEG

As in Part II with the addition that instead of the averaging of the whole period of EEG recording (i.e. 24h) for individual animals , average was done for every minute for individual animal and used later for statistical analysis.

Statistical analysis, as in the previous study, was done with GraphPrism v.7 (GraphPad Software Inc., CA, USA) and Matlab (MathWorks, USA). The one-way ANOVA test followed by multiple comparisons was used for the test of significance. The level of significance was defined as $p < 0.05$.

Next, for every animal we calculated an average total power for each EEG-band of a baseline EEG for each EEG-band. And corresponding average value was used later for the normalization of the MS and Pilo signal.

ERP

As in Part II.

Pilocarpine challenge

Resemble the protocol for the induction of the TLE in rodents. For the pilocarpine challenge subclinical dose of the pilocarpine was used (260 mg/kg) instead of 300 mg/kg. 30 minutes prior to the pilocarpine mice were injected with methylscopolamine. Animals were connected for the EEG recording and the clinical state was controlled visually for 160 min. Animals who did not enter to the SE within 120 min received an additional dose of the pilocarpine (260 mg/kg).

Results

Perspective outcome of PILO challenge in MB receivers

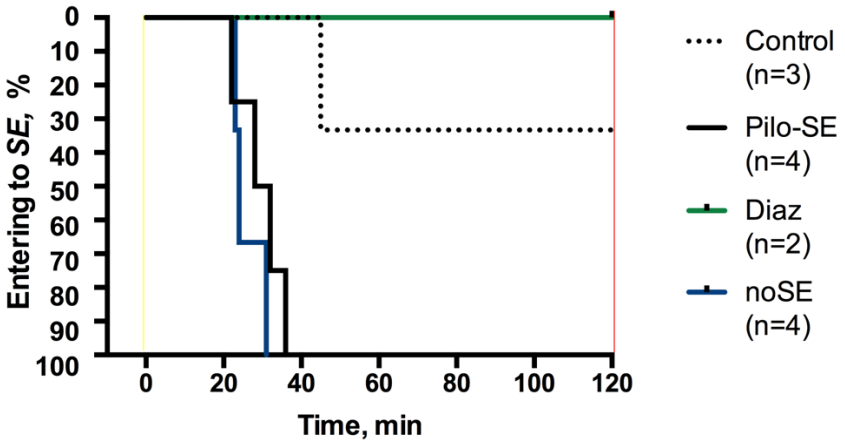

Figure 15. Survival curves in response to the pilocarpine challenge of the first (pilot group). Curves on the graph represent the corresponding groups: Control (dotted black line), Pilo-SE (black line), Diazepam (green) and Pilo-noSE (blue). Axis Y represents percentage of animals entered to SE, X - time in minutes. Yellow line indicates the time of the pilocarpine injection, red one - end of the observation. Comparison of the curves showed significant difference between Control and Pilo-SE groups (Logrank test, Chi square 11.95, p = 0.0076), and Control vs Pilo-noSE group (Logrank test, Chi square 5.052, p = 0.0246).

EEG: occurrence of seizures and power spectral density analysis

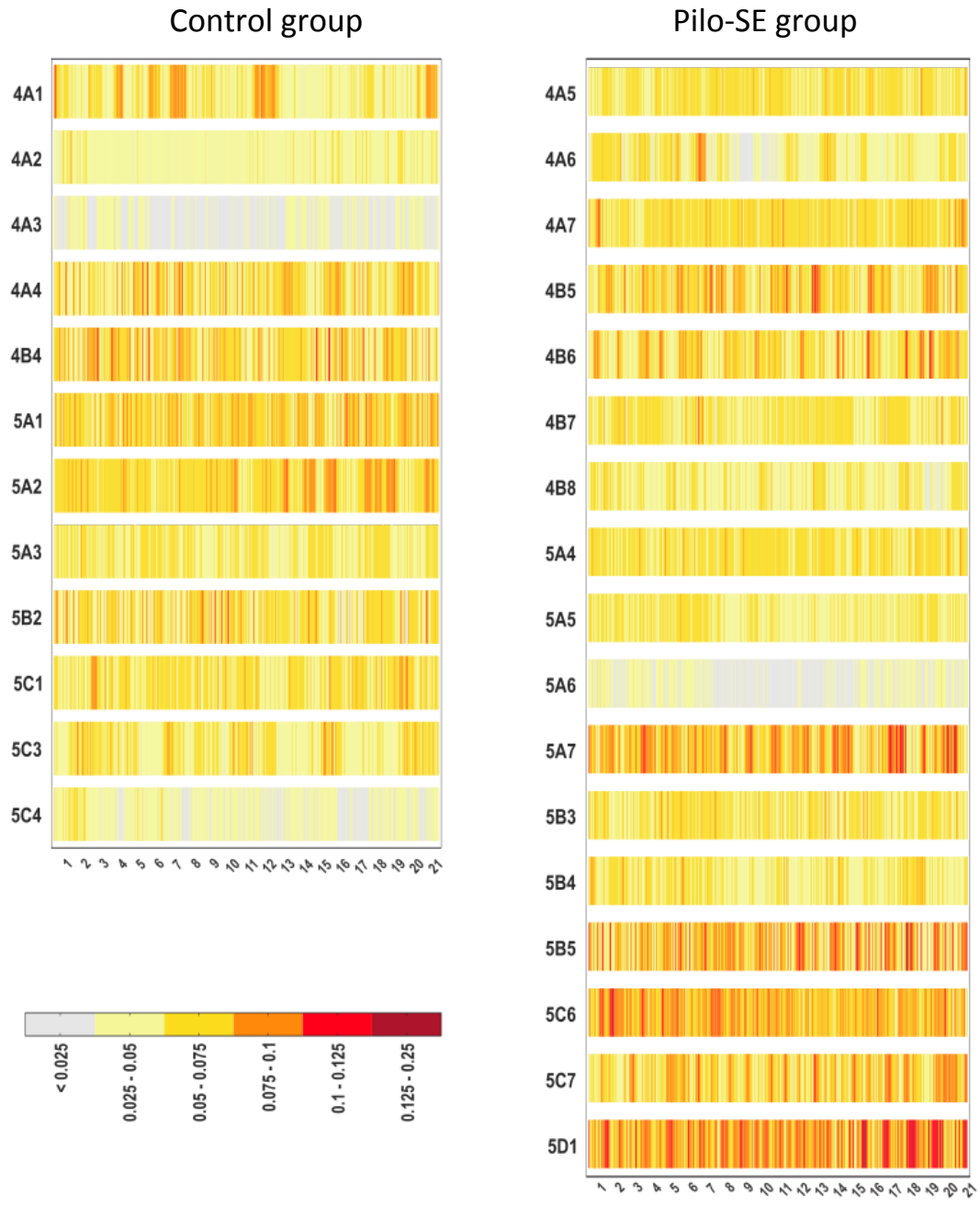

Figure 16. Representation of the gamma-band in 24h EEG recording.
Profile of time changes in the relative gamma (25-70 Hz) total power in the sensorimotor cortex of receivers of the microbiota.
Each row represents individual animal and X axis indicate time in hours. Changes in gamma visualized in colored scale (from grey as minimal to maximal - brown) as a ratio to the total power of all frequency bands for every epoch of analysis for every hour of the EEG recording.

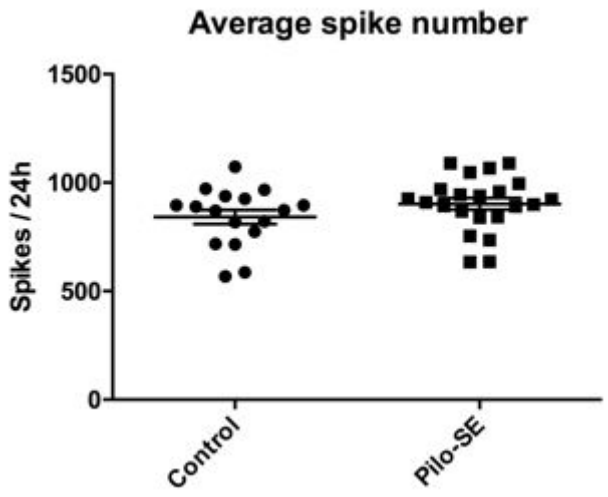

Figure 17. Spikes counting in the 24 hours of the EEG recording.
Appearance of the spikes in the sensory-motor cortex of animals inoculated with microbiota (Mean ± SEM).
Axis Y represents average spikes-count calculated in the 24 hours of EEG monitoring. Axis X indicate experimental groups.

Auditory ERPs

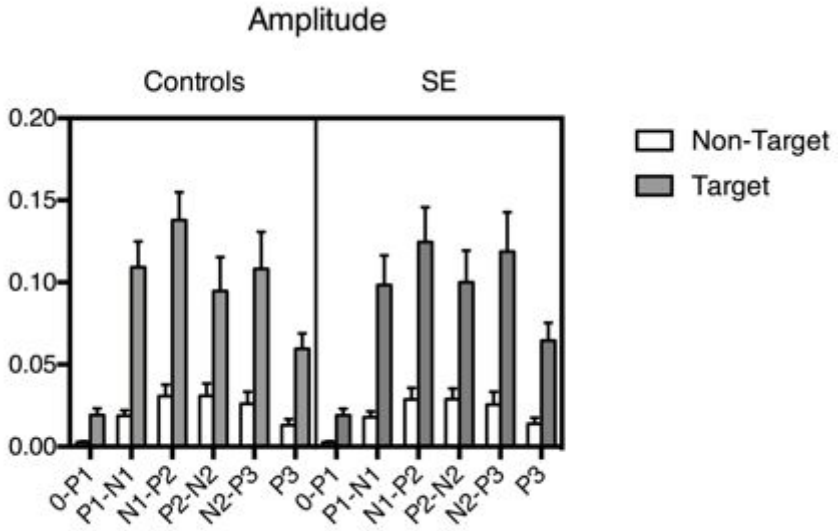

Figure 18. Amplitude of the auditory evoked potentials in the sensory-motor cortex of receivers of the microbiota (Mean+SEM).
White bars represent non-target stimuli (4 kHz), grey bars - target (8 kHz). Axis Y represents amplitude of the evoked responses in mV. On the axis X - grouped columns corresponding to the ERP peaks.
Black vertical line separate experimental groups.

Pilocarpine challenge

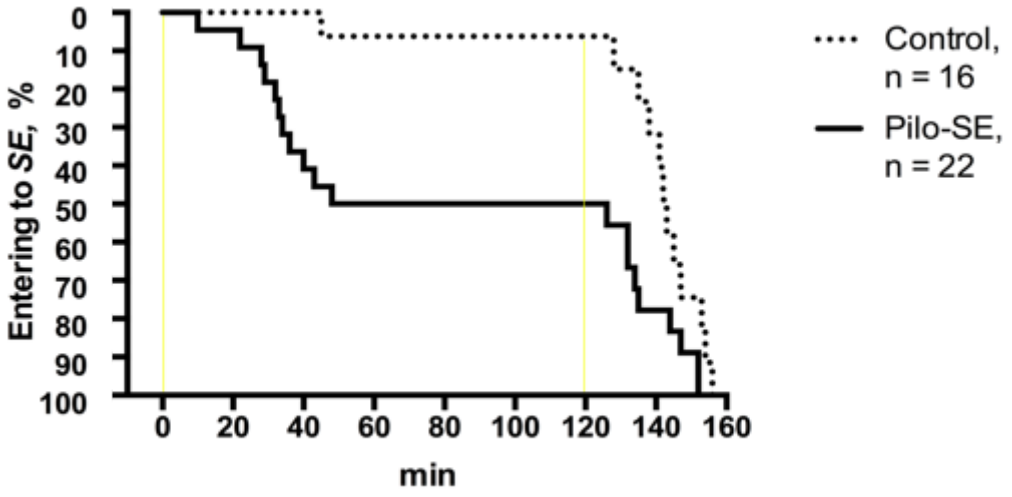

Figure 19. Survival curves in response to the pilocarpine challenge.
Curves on the graph represent the corresponding groups: Control (dotted black line) and Pilo-SE (black line). Axis Y represents percentage of animals entered to SE, X - time in minutes. Yellow line indicates the time of the pilocarpine injection, in the time point of 120 minutes animals who did not enter to the SE received an additional dose of the pilocarpine.
Comparison of the curves showed significant difference between two groups (Logrank test, Chi square 7.823, p = 0.0052).

Discussion

For the present study so-called “virtually germ free” mouse model was chosen. This refers to the depletion of the host microbiota with the mix of antibiotics that cover wide spectrum of bacterial species. This model has its limitations and as the complete germ-free status is not obtained, the variation in the survived species can be observed. On the other hand, GF mice, as a potential model to study epilepsy, have an abnormal baseline and lack of the early-life immune priming which is crucial for the normal development of the immune system. What is more, even brief postnatal GF period changes levels of systemic regulatory immune cells and cytokine production (C. H. F. Hansen et al., 2012). As it was described before, immune signalling molecules can play an important role in the microbiota-gut-brain interaction, thus “virtually germ free” mouse model, taking in consideration all its limitations, appears to be more appropriate to study possible impact of the microbiota on the excitability of the brain.

Results of the pilocarpine challenge in the first (pilot) group suggested the possibility that pro-pathological microbiota obtained from the Pilo-SE donors may lead to the lowering of the seizures threshold in recipients. Interestingly, pups inoculated with Pilo-noSE microbiota (from donors who did not enter to the SE after the injection of the pilocarpine, but in Part II were shown to have seizures in the hippocampus) also were more susceptible in the challenge with the subclinical dose of the pilocarpine. As the number of the animals in each group was limited, additional runs of the experiment were performed.

In the next runs we decided to concentrate on the main experimental groups: Pilo-SE and Control.

After the inoculation with the microbiota of interest pups were monitored for the detection of spontaneous seizures. Visual observation, together with the electrophysiological data (EEG, ERP) reveals that:

- Inoculation with the pro-pathological microbiota was not sufficient to provoke behavioral manifestations of the seizure-like activity.
- Baseline EEG recording reveal slight alterations in the presence of gamma band in individual animals. Gamma waves (25-70 Hz), representing high frequency activity of the brain, usually indicate the pathological state of the brain (altered excitability which is leading to the lowering seizures threshold).
- Average amount of spikes in mice inoculated with the pro-pathological microbiota was the same as in control group, which leads to the idea that inoculation with the pro-pathological microbiota *per se* was not sufficient to elucidate the differences in the groups. The presence of the spikes in control groups can be due to the surgical implantation of the electrodes which by itself might cause a microinflammation.
- Evaluation of the respond to the auditory stimuli did not reveal any significant functional differences between two groups (Pilo-SE vs Control).
- During challenging animals with the pilocarpine, mice who received microbiota from the Pilo-SE donors appeared to have lower threshold for the seizure activity. This suggests that mice received “pro-pathological” microbiota have compromised excitability considering that subclinical dose lead to developing SE.

In this study epilepsy was used as a hyperexcitability paradigm. Thus, challenging mice with the subclinical dose of pilocarpine (260 mg/kg instead of 300) is mimicking any challenge for immune system that can spontaneously occur (fever, flu, etc.)

The data of the present study suggests that the microbiological background of the host can have an important role in the response of the organism to the events challenging the excitability of the brain.

This supports the growing body of literature that underlines the crucial role of the gut microbiota in development, progression and even improvement of the CNS pathologies including depression, stroke (Winek, Dirnagl, & Meisel, 2016) and autism (Kang et al., 2017).

Conclusion

The idea of the microbiota-gut-brain interaction is relatively young and constantly developing. Great breakthrough has happened recently and is happening now, but there is still a lot to discover.

In the studies described above we aimed to investigate the possible impact of the microbiota obtained from the donors - mouse model of TLE on the brain excitability of the healthy recipients. Data presented here suggest that animals who received “pro-pathological” microbiota have compromised excitability, considering that this group appeared to have lower threshold for developing seizure activity. Microbiota composition of the donors with induced TLE was characterised by the increase in *Sutterella, Prevotella, Dorea, Coprobacillus* and *Candidatus Arthromitus* in comparison with the baseline, what hypothetically, through the GBA, might have an effect on the excitability of the brain and subsequently on the threshold for the development of the seizures.

To some point, the idea that seizure activity can be moderated by the gastroenterological interventions is not new. Ketogenic diet (KD) is known to be an effective treatment for the drug-resistant epilepsy in children and to provide beneficial effect in adults (Liu et al., 2018). But only recently intestinal microbiota was recognized as the active contributor to this effect. New findings suggest that in infants with diagnosed epilepsy, after following KD, bacterial patterns were significantly changed and, what is more, changes in microbiota composition correlated with the reduction in the seizure activity (Xie et al., 2017).

Nowadays the growing body of literature suggests that patients with various neurological disorders can potentially benefit from the interventions regarding intestinal microbiota.

For instance, it was shown that in animal model of MS, administration of human commensal bacterium *P. histicola* provide evident immunomodulatory, anti-inflammatory response and reduce demyelination, thus may be considered for treatment strategies for MS (Mangalam et al., 2017). Recent issue of the Neurotherapeutics (January 2018) was completely dedicated to the role of the microbiome in neurologic diseases. It covers explicitly the intersections between MS and the state of the microbiota community in the gut, raising a lot of open-questions, and, at the same time, underlying the importance of considering this “forgotten organ” in the incidence and progression of the MS and possibilities for the management of the disease outcome.

Also, in the recent review regarding the role of the intestinal microbiome in ASD, potential therapeutic strategies such as probiotic treatment and FMT were highlighted (Mangalam et al., 2017). In the mouse model of ASD, KD was shown to modulate the microbiota composition and potentially contribute to alleviation of neurological symptoms associated with the disease (Newell et al., 2016). Another studies performed on the animal model showed that administration of the human commensal bacteria (*Bacteroides fragilis*) regulates gut permeability, influences microbial composition and improves ASD-related behaviors (Hsiao et al., 2013).

In the case of AD, nutritional interventions were shown to influence the composition of the gut microbiota and subsequently affect the formation and aggregation of cerebral amyloid-β, several “antiamyloidogenic” and

“proamyloidogenic” products were identified for the targeted personalized nutrition protocols in patients with AD (Pistollato et al., 2016).

Also, several probiotics were reported to improve memory deficit in experimental models (Athari Nik Azm et al., 2018; Nimgampalle & Kuna, 2017) and to alleviate AD-related impairments in randomized clinical trial as well (Akbari et al., 2016).

To summarize, evidence from both experimental and clinical research suggests that probiotics, prebiotics, FMT and nutrition-based therapies targeting gut-brain axis may become a safe and powerful support for the patients with the incurable forms of neurologic diseases . Thus, to elucidate exact pathways of microbiota-gut-brain interaction for targeted and effective therapeutic strategies, further investigations in this promising and exciting field are strongly needed.

References

Aguilar, J., Urday-cornejo, V., Donabedian, S., Perri, M., Tibbetts, R., & Zervos, M. (2010). *staphylococcus aureus*: Case Series and Literature Review Meningitis: Case Series and Literature Review. *Medicine*, *89*(2), 117–125. https://doi.org/10.1097/MD.0b013e3181d5453d

Ahmed, I., Yokota, A., Yamazoe, A., & Fujiwara, T. (2007). Proposal of Lysinibacillus boronitolerans gen. nov. sp. nov., and transfer of Bacillus fusiformis to Lysinibacillus fusiformis comb. nov. and Bacillus sphaericus to Lysinibacillus sphaericus comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, *57*(Pt 5), 1117–1125. https://doi.org/10.1099/ijs.0.63867-0

Akbari, E., Asemi, Z., Daneshvar Kakhaki, R., Bahmani, F., Kouchaki, E., Tamtaji, O. R., … Salami, M. (2016). Effect of Probiotic Supplementation on Cognitive Function and Metabolic Status in Alzheimer's Disease: A Randomized, Double-Blind and Controlled Trial. *Frontiers in Aging Neuroscience*, *8*, 256. https://doi.org/10.3389/fnagi.2016.00256

Alam, R., Abdolmaleky, H. M., & Zhou, J.-R. (2017). Microbiome, inflammation, epigenetic alterations, and mental diseases. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics: The Official Publication of the International Society of Psychiatric Genetics*, *174*(6), 651–660. https://doi.org/10.1002/ajmg.b.32567

Arnott, S. R., Bardouille, T., Ross, B., & Alain, C. (2011). Neural generators underlying concurrent sound segregation. *Brain Research*, *1387*, 116–124. https://doi.org/10.1016/j.brainres.2011.02.062

Athari Nik Azm, S., Djazayeri, A., Safa, M., Azami, K., Ahmadvand, B., Sabbaghziarani, F., …

Vafa, M. (2018). Lactobacillus and Bifidobacterium Ameliorate Memory and Learning Deficits and oxidative stress in A β (1-42) Injected Rats. *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquee, Nutrition et Metabolisme*. https://doi.org/10.1139/apnm-2017-0648

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., … Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(44), 15718–15723. https://doi.org/10.1073/pnas.0407076101

Barko, P. C., McMichael, M. A., Swanson, K. S., & Williams, D. A. (2017). The Gastrointestinal Microbiome: A Review. *Journal of Veterinary Internal Medicine / American College of Veterinary Internal Medicine*. https://doi.org/10.1111/jvim.14875

Bartolomei, F., Khalil, M., Wendling, F., Sontheimer, A., Régis, J., Ranjeva, J.-P., … Chauvel, P. (2005). Entorhinal cortex involvement in human mesial temporal lobe epilepsy: an electrophysiologic and volumetric study. *Epilepsia*, *46*(5), 677–687. https://doi.org/10.1111/j.1528-1167.2005.43804.x

Benakis, C., Brea, D., Caballero, S., Faraco, G., Moore, J., Murphy, M., … Anrather, J. (2016). Commensal microbiota affects ischemic stroke outcome by regulating intestinal $\gamma\delta$ T cells. *Nature Medicine*, *22*, 516. https://doi.org/10.1038/nm.4068

Bercik, P., Collins, S. M., & Verdu, E. F. (2012). Microbes and the gut-brain axis. *Neurogastroenterology & Motility*, *24*(5), 405–413. https://doi.org/10.1111/j.1365-2982.2012.01906.x

Berer, K., Gerdes, L. A., Cekanaviciute, E., Jia, X., Xiao, L., Xia, Z., … Wekerle, H. (2017). Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proceedings of the National Academy of Sciences of the*

United States of America, *114*(40), 10719–10724. https://doi.org/10.1073/pnas.1711233114

Bertini, G., Bramanti, P., Constantin, G., Pellitteri, M., Radu, B. M., Radu, M., & Fabene, P. F. (2013). New players in the neurovascular unit: Insights from experimental and clinical epilepsy. *Neurochemistry International*, *63*(7), 652–659. https://doi.org/10.1016/j.neuint.2013.08.001

Blackshaw, L. A., Brookes, S. J. H., Grundy, D., & Schemann, M. (2007). Sensory transmission in the gastrointestinal tract. *Neurogastroenterology and Motility: The Official Journal of the European Gastrointestinal Motility Society*, *19*(1 Suppl), 1–19. https://doi.org/10.1111/j.1365-2982.2006.00871.x

Blackwood, D. H., & Muir, W. J. (1990). Cognitive brain potentials and their application. *The British Journal of Psychiatry. Supplement*, (9), 96–101. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/2291824

Bleich, A., & Hansen, A. K. (2012). Time to include the gut microbiota in the hygienic standardisation of laboratory rodents. *Comparative Immunology, Microbiology and Infectious Diseases*, *35*(2), 81–92. https://doi.org/10.1016/j.cimid.2011.12.006

Bradley, W. G., & Shey, R. B. (2000). MR imaging evaluation of seizures. *Radiology*, *214*(3), 651–656. https://doi.org/10.1148/radiology.214.3.r00mr42651

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Tóth, M., … Pettersson, S. (2014). The gut microbiota influences blood-brain barrier permeability in mice. *Science Translational Medicine*, *6*(263), 263ra158. https://doi.org/10.1126/scitranslmed.3009759

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., … Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central

GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(38), 16050–16055. https://doi.org/10.1073/pnas.1102999108

Brouland, J. P., Sala, N., Tusgul, S., Rebecchini, C., & Kovari, E. (2018). Bacillus cereus bacteremia with central nervous system involvement: A neuropathological study. *Clinical Neuropathology*, *37*(1), 22–27. https://doi.org/10.5414/NP301041

Calcaterra, N. E., & Barrow, J. C. (2014). Classics in chemical neuroscience: diazepam (valium). *ACS Chemical Neuroscience*, *5*(4), 253–260. https://doi.org/10.1021/cn5000056

Cantarel, B. L., Waubant, E., Chehoud, C., Kuczynski, J., DeSantis, T. Z., Warrington, J., ⋯ Mowry, E. M. (2015). Gut microbiota in multiple sclerosis: possible influence of immunomodulators. *Journal of Investigative Medicine: The Official Publication of the American Federation for Clinical Research*, *63*(5), 729–734. https://doi.org/10.1097/JIM.0000000000000192

Caravaglios, G., Natalè, E., Ferraro, G., Fierro, B., Raspanti, G., & Daniele, O. (2001). Auditory event-related potentials (P300) in epileptic patients. *Neurophysiologie Clinique = Clinical Neurophysiology*, *31*(2), 121–129. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11433674

Catanzaro, R., Anzalone, M., Calabrese, F., Milazzo, M., Capuana, M., Italia, A., ⋯ Marotta, F. (2015). The gut microbiota and its correlations with the central nervous system disorders. *Panminerva Medica*, *57*(3), 127–143. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/25390799

Chang, W. N., Lu, C. H., Huang, C. R., & Chuang, Y. C. (2000). Community-acquired Acinetobacter meningitis in adults. *Infection*, *28*(6), 395–397. Retrieved from

https://www.ncbi.nlm.nih.gov/pubmed/11139162

Cho, I., & Blaser, M. J. (2012). The human microbiome: at the interface of health and disease. *Nature Reviews. Genetics*, *13*(4), 260–270. https://doi.org/10.1038/nrg3182

Chow, J., Lee, S. M., Shen, Y., Khosravi, A., & Mazmanian, S. K. (2010). Host-bacterial symbiosis in health and disease. *Advances in Immunology*, *107*, 243–274. https://doi.org/10.1016/B978-0-12-381300-8.00008-3

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R. D., Shanahan, F., ⋯ Cryan, J. F. (2013). The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Molecular Psychiatry*, *18*(6), 666–673. https://doi.org/10.1038/mp.2012.77

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I., & Knight, R. (2009). Bacterial community variation in human body habitats across space and time. *Science*, *326*(5960), 1694–1697. https://doi.org/10.1126/science.1177486

Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews. Neuroscience*, *13*(10), 701–712. https://doi.org/10.1038/nrn3346

Curia, G., Longo, D., Biagini, G., Jones, R. S. G., & Avoli, M. (2008). The pilocarpine model of temporal lobe epilepsy. *Journal of Neuroscience Methods*, *172*(2), 143–157. https://doi.org/10.1016/j.jneumeth.2008.04.019

De Palma, G., Blennerhassett, P., Lu, J., Deng, Y., Park, A. J., Green, W., ⋯ Bercik, P. (2015). Microbiota and host determinants of behavioural phenotype in maternally separated mice. *Nature Communications*, *6*, 7735. https://doi.org/10.1038/ncomms8735

Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., ⋯ Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior.

Proceedings of the National Academy of Sciences of the United States of America, *108*(7), 3047–3052. https://doi.org/10.1073/pnas.1010529108

Dinan, T. G., Borre, Y. E., & Cryan, J. F. (2014). Genomics of schizophrenia: time to consider the gut microbiome? *Molecular Psychiatry*, *19*(12), 1252–1257. https://doi.org/10.1038/mp.2014.93

Ding, H. T., Taur, Y., & Walkup, J. T. (2017). Gut Microbiota and Autism: Key Concepts and Findings. *Journal of Autism and Developmental Disorders*, *47*(2), 480–489. https://doi.org/10.1007/s10803-016-2960-9

Dubé, C. M., Brewster, A. L., Richichi, C., Zha, Q., & Baram, T. Z. (2007). Fever, febrile seizures and epilepsy. *Trends in Neurosciences*, *30*(10), 490–496. https://doi.org/10.1016/j.tins.2007.07.006

Duerkop, B. A., Vaishnava, S., & Hooper, L. V. (2009). Immune responses to the microbiota at the intestinal mucosal surface. *Immunity*, *31*(3), 368–376. https://doi.org/10.1016/j.immuni.2009.08.009

Eren, A. M., Borisy, G. G., Huse, S. M., & Mark Welch, J. L. (2014). Oligotyping analysis of the human oral microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(28), E2875–E2884. https://doi.org/10.1073/pnas.1409644111

Fabene, P. F., Bramanti, P., & Constantin, G. (2010). The emerging role for chemokines in epilepsy. *Journal of Neuroimmunology*, *224*(1-2), 22–27. https://doi.org/10.1016/j.jneuroim.2010.05.016

Fabene, P. F., Laudanna, C., & Constantin, G. (2013). Leukocyte trafficking mechanisms in epilepsy. *Molecular Immunology*, *55*(1), 100–104. https://doi.org/10.1016/j.molimm.2012.12.009

Fabene, P. F., Mora, G. N., Martinello, M., Rossi, B., Merigo, F., Ottoboni, L., … Constantin, G. (2008). A role for leukocyte-endothelial adhesion mechanisms in epilepsy. *Nature Medicine*, *14*(12), 1377–1383. https://doi.org/10.1038/nm.1878

Falk, P. G., Hooper, L. V., Midtvedt, T., & Gordon, J. I. (1998). Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiology and Molecular Biology Reviews: MMBR*, *62*(4), 1157–1170. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9841668

Fisher, R. S., Cross, J. H., French, J. A., Higurashi, N., Hirsch, E., Jansen, F. E., … Zuberi, S. M. (2017). Operational classification of seizure types by the International League Against Epilepsy: Position Paper of the ILAE Commission for Classification and Terminology. *Epilepsia*, *58*(4), 522–530. https://doi.org/10.1111/epi.13670

Forbes, J. D., Van Domselaar, G., & Bernstein, C. N. (2016). The Gut Microbiota in Immune-Mediated Inflammatory Diseases. *Frontiers in Microbiology*, *7*, 1081. https://doi.org/10.3389/fmicb.2016.01081

Foster, J. A., & McVey Neufeld, K.-A. (2013). Gut-brain axis: how the microbiome influences anxiety and depression. *Trends in Neurosciences*, *36*(5), 305–312. https://doi.org/10.1016/j.tins.2013.01.005

Freedman, S. N., Shahi, S. K., & Mangalam, A. K. (2017). The “Gut Feeling”: Breaking Down the Role of Gut Microbiome in Multiple Sclerosis. *Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics*. https://doi.org/10.1007/s13311-017-0588-x

Fukai, M., Motomura, N., Kobayashi, S., Asaba, H., & Sakai, T. (1990). Event-related potential (P300) in epilepsy. *Acta Neurologica Scandinavica*, *82*(3), 197–202. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/2125399

Furness, J. B. (2006). The organisation of the autonomic nervous system: peripheral connections. *Autonomic Neuroscience: Basic & Clinical*, *130*(1-2), 1–5. https://doi.org/10.1016/j.autneu.2006.05.003

Gareau, M. G., Wine, E., Rodrigues, D. M., Cho, J. H., Whary, M. T., Philpott, D. J., ⋯ Sherman, P. M. (2011). Bacterial infection causes stress-induced memory dysfunction in mice. *Gut*, *60*(3), 307–317. https://doi.org/10.1136/gut.2009.202515

Gershon, M. D. (1999). *The second brain: a groundbreaking new understanding of nervous disorders of the stomach and intestine* (p. 320). HarperPerennial.

Golubeva, A. V., Crampton, S., Desbonnet, L., Edge, D., O'Sullivan, O., Lomasney, K. W., ⋯ Cryan, J. F. (2015). Prenatal stress-induced alterations in major physiological systems correlate with gut microbiota composition in adulthood. *Psychoneuroendocrinology*, *60*, 58–74. https://doi.org/10.1016/j.psyneuen.2015.06.002

Hague, A., Singh, B., & Paraskeva, C. (1997). Butyrate acts as a survival factor for colonic epithelial cells: further fuel for the in vivo versus in vitro debate. *Gastroenterology*, *112*(3), 1036–1040. https://doi.org/10.1053/gast.1997.v112.agast971036

Hansen, A. K., Friis Hansen, C. H., Krych, L., & Nielsen, D. S. (2014). Impact of the gut microbiota on rodent models of human disease. *World Journal of Gastroenterology: WJG*, *20*(47), 17727–17736. https://doi.org/10.3748/wjg.v20.i47.17727

Hansen, C. H. F., Nielsen, D. S., Kverka, M., Zakostelska, Z., Klimesova, K., Hudcovic, T., ⋯ Hansen, A. K. (2012). Patterns of early gut colonization shape future immune responses of the host. *PloS One*, *7*(3), e34043. https://doi.org/10.1371/journal.pone.0034043

Herman, J. P., McKlveen, J. M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., ⋯ Myers, B. (2016). Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response. *Comprehensive Physiology*, *6*(2), 603–621. https://doi.org/10.1002/cphy.c150015

Hesdorffer, D. C., Logroscino, G., Cascino, G., Annegers, J. F., & Hauser, W. A. (1998). Risk of unprovoked seizure after acute symptomatic seizure: effect of status epilepticus. *Annals of Neurology*, *44*(6), 908–912. https://doi.org/10.1002/ana.410440609

Hoban, A. E., Stilling, R. M., Ryan, F. J., Shanahan, F., Dinan, T. G., Claesson, M. J., … Cryan, J. F. (2016). Regulation of prefrontal cortex myelination by the microbiota. *Translational Psychiatry*, *6*(4), e774. https://doi.org/10.1038/tp.2016.42

Hsiao, E. Y., McBride, S. W., Hsien, S., Sharon, G., Hyde, E. R., McCue, T., … Mazmanian, S. K. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell*, *155*(7), 1451–1463. https://doi.org/10.1016/j.cell.2013.11.024

Huang, C.-H., Shen, C.-C., Liang, Y.-C., & Jan, T.-R. (2016). The probiotic activity of Lactobacillus murinus against food allergy. *Journal of Functional Foods*, *25*, 231–241. https://doi.org/10.1016/j.jff.2016.06.006

Jiao, N., Baker, S. S., Nugent, C. A., Tsompana, M., Cai, L., Wang, Y., … Zhu, L. (2018). Gut microbiome may contribute to insulin resistance and systemic inflammation in obese rodents: a meta-analysis. *Physiological Genomics*, *50*(4), 244–254. https://doi.org/10.1152/physiolgenomics.00114.2017

Kang, D.-W., Adams, J. B., Gregory, A. C., Borody, T., Chittick, L., Fasano, A., … Krajmalnik-Brown, R. (2017). Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study. *Microbiome*, *5*(1), 10. https://doi.org/10.1186/s40168-016-0225-7

Kang, D.-W., Park, J. G., Ilhan, Z. E., Wallstrom, G., Labaer, J., Adams, J. B., & Krajmalnik-Brown, R. (2013). Reduced incidence of Prevotella and other fermenters in intestinal microflora of autistic children. *PloS One*, *8*(7), e68322.

https://doi.org/10.1371/journal.pone.0068322

Karlócai, M. R., Tóth, K., Watanabe, M., Ledent, C., Juhász, G., Freund, T. F., & Maglóczky, Z. (2011). Redistribution of CB1 cannabinoid receptors in the acute and chronic phases of pilocarpine-induced epilepsy. *PloS One*, *6*(11), e27196. https://doi.org/10.1371/journal.pone.0027196

Kolínská, R., Spanělová, P., Dřevínek, M., Hrabák, J., & Zemličková, H. (2015). Species identification of strains belonging to genus Citrobacter using the biochemical method and MALDI-TOF mass spectrometry. *Folia Microbiologica*, *60*(1), 53–59. https://doi.org/10.1007/s12223-014-0340-4

Kutas, M., McCarthy, G., & Donchin, E. (1977). Augmenting mental chronometry: the P300 as a measure of stimulus evaluation time. *Science*, *197*(4305), 792–795. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/887923

Kwa, S.-F., Beverley, P., & Smith, A. L. (2006). Peyer's patches are required for the induction of rapid Th1 responses in the gut and mesenteric lymph nodes during an enteric infection. *Journal of Immunology* , *176*(12), 7533–7541. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/16751400

Ley, R. E., Peterson, D. A., & Gordon, J. I. (2006). Ecological and Evolutionary Forces Shaping Microbial Diversity in the Human Intestine. *Cell*, *124*(4), 837–848. https://doi.org/10.1016/j.cell.2006.02.017

Liang, X., & FitzGerald, G. A. (2017). Timing the Microbes: The Circadian Rhythm of the Gut Microbiome. *Journal of Biological Rhythms*, *32*(6), 505–515. https://doi.org/10.1177/0748730417729066

Liégeois-Chauvel, C., Musolino, A., Badier, J. M., Marquis, P., & Chauvel, P. (1994). Evoked potentials recorded from the auditory cortex in man: evaluation and topography of the

middle latency components. *Electroencephalography and Clinical Neurophysiology*, *92*(3), 204–214. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/7514990

Li, G., Bauer, S., Nowak, M., Norwood, B., Tackenberg, B., Rosenow, F., ⋯ Hamer, H. M. (2011). Cytokines and epilepsy. *Seizure: The Journal of the British Epilepsy Association*, *20*(3), 249–256. https://doi.org/10.1016/j.seizure.2010.12.005

Liu, H., Yang, Y., Wang, Y., Tang, H., Zhang, F., Zhang, Y., & Zhao, Y. (2018). Ketogenic diet for treatment of intractable epilepsy in adults: A meta-analysis of observational studies. *Epilepsia Open*, *3*(1), 9–17. https://doi.org/10.1002/epi4.12098

Löscher, W., & Potschka, H. (2005). Drug resistance in brain diseases and the role of drug efflux transporters. *Nature Reviews. Neuroscience*, *6*(8), 591–602. https://doi.org/10.1038/nrn1728

Luckey, T. D. (1972). Introduction to intestinal microecology. *The American Journal of Clinical Nutrition*, *25*(12), 1292–1294. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/4639749

Luck, S. J. (2012). Event-related potentials. In H. Cooper, P. M. Camic, D. L. Long, A. T. Panter, D. Rindskopf & K. J. Sher (Ed.), *APA Handbook of Research Methods in Psychology: Volume 1, Foundations, Planning, Measures, and Psychometrics.* Washington,DC: APA. Retrieved from http://lucklab.ucdavis.edu/uploads/5/8/4/6/58469631/luck_2012_apa_handbook.pdf

Ludbrook, J. (2008). Analysis of 2 x 2 tables of frequencies: matching test to experimental design. *International Journal of Epidemiology*, *37*(6), 1430–1435. https://doi.org/10.1093/ije/dyn162

Lundberg, R., Bahl, M. I., Licht, T. R., Toft, M. F., & Hansen, A. K. (2017). Microbiota composition of simultaneously colonized mice housed under either a gnotobiotic

isolator or individually ventilated cage regime. *Scientific Reports*, *7*, 42245. https://doi.org/10.1038/srep42245

Lyte, M. (2010). The microbial organ in the gut as a driver of homeostasis and disease. *Medical Hypotheses*, *74*(4), 634–638. https://doi.org/10.1016/j.mehy.2009.10.025

Mancuso, C., & Santangelo, R. (2017). Alzheimer's disease and gut microbiota modifications: The long way between preclinical studies and clinical evidence. *Pharmacological Research: The Official Journal of the Italian Pharmacological Society*. https://doi.org/10.1016/j.phrs.2017.12.009

Mangalam, A., Shahi, S. K., Luckey, D., Karau, M., Marietta, E., Luo, N., ⋯ Murray, J. (2017). Human Gut-Derived Commensal Bacteria Suppress CNS Inflammatory and Demyelinating Disease. *Cell Reports*, *20*(6), 1269–1277. https://doi.org/10.1016/j.celrep.2017.07.031

Marchi, N., & Lerner-Natoli, M. (2013). Cerebrovascular remodeling and epilepsy. *The Neuroscientist: A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *19*(3), 304–312. https://doi.org/10.1177/1073858412462747

Marchi, N., Teng, Q., Ghosh, C., Fan, Q., Nguyen, M. T., Desai, N. K., ⋯ Janigro, D. (2010). Blood-brain barrier damage, but not parenchymal white blood cells, is a hallmark of seizure activity. *Brain Research*, *1353*, 176–186. https://doi.org/10.1016/j.brainres.2010.06.051

Mathern, G. W., Adelson, P. D., Cahan, L. D., & Leite, J. P. (2002). Hippocampal neuron damage in human epilepsy: Meyer's hypothesis revisited. *Progress in Brain Research*, *135*, 237–251. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/12143344

Mayer, E. A., Tillisch, K., & Gupta, A. (2015). Gut/brain axis and the microbiota. *The Journal of Clinical Investigation*, *125*(3), 926–938. https://doi.org/10.1172/JCI76304

Mazzuferi, M., Kumar, G., Rospo, C., & Kaminski, R. M. (2012). Rapid epileptogenesis in the mouse pilocarpine model: Video-EEG, pharmacokinetic and histopathological characterization. *Experimental Neurology*, *238*(2), 156–167. https://doi.org/10.1016/j.expneurol.2012.08.022

Milesi, S., Boussadia, B., Plaud, C., Catteau, M., Rousset, M.-C., De Bock, F., ⋯ Marchi, N. (2014). Redistribution of PDGFR β cells and NG2DsRed pericytes at the cerebrovasculature after status epilepticus. *Neurobiology of Disease*, *71*, 151–158. https://doi.org/10.1016/j.nbd.2014.07.010

Montaña, S., Schramm, S. T. J., Traglia, G. M., Chiem, K., Parmeciano Di Noto, G., Almuzara, M., ⋯ Ramírez, M. S. (2016). The Genetic Analysis of an Acinetobacter johnsonii Clinical Strain Evidenced the Presence of Horizontal Genetic Transfer. *PloS One*, *11*(8), e0161528. https://doi.org/10.1371/journal.pone.0161528

Moshé, S. L., Perucca, E., Ryvlin, P., & Tomson, T. (2015). Epilepsy: new advances. *The Lancet*, *385*(9971), 884–898. https://doi.org/10.1016/S0140-6736(14)60456-6

Nagy, N., & Goldstein, A. M. (2017). Enteric nervous system development: A crest cell's journey from neural tube to colon. *Seminars in Cell & Developmental Biology*, *66*, 94–106. https://doi.org/10.1016/j.semcdb.2017.01.006

Navarro Mora, G., Bramanti, P., Osculati, F., Chakir, A., Nicolato, E., Marzola, P., ⋯ Fabene, P. F. (2009). Does pilocarpine-induced epilepsy in adult rats require status epilepticus? *PloS One*, *4*(6). https://doi.org/10.1371/journal.pone.0005759

Neufeld, K. M., Kang, N., Bienenstock, J., & Foster, J. a. (2011). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology & Motility*, *23*(3), 255–e119. https://doi.org/10.1111/j.1365-2982.2010.01620.x

Newell, C., Bomhof, M. R., Reimer, R. A., Hittel, D. S., Rho, J. M., & Shearer, J. (2016).

Ketogenic diet modifies the gut microbiota in a murine model of autism spectrum disorder. *Molecular Autism*, *7*(1), 37. https://doi.org/10.1186/s13229-016-0099-3

Nimgampalle, M., & Kuna, Y. (2017). Anti-Alzheimer Properties of Probiotic,Lactobacillus plantarumMTCC 1325 in Alzheimer's Disease induced Albino Rats. *Journal of Clinical and Diagnostic Research: JCDR*, *11*(8), KC01–KC05. https://doi.org/10.7860/JCDR/2017/26106.10428

O'Hara, A. M., & Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Reports*, *7*(7), 688–693. https://doi.org/10.1038/sj.embor.7400731

Ohkawara, S., Furuya, H., Nagashima, K., Asanuma, N., & Hino, T. (2006). Effect of oral administration of Butyrivibrio fibrisolvens MDT-1 on experimental enterocolitis in mice. *Clinical and Vaccine Immunology: CVI*, *13*(11), 1231–1236. https://doi.org/10.1128/CVI.00267-06

O'Mahony, S. M., Hyland, N. P., Dinan, T. G., & Cryan, J. F. (2011). Maternal separation as a model of brain-gut axis dysfunction. *Psychopharmacology*, *214*(1), 71–88. https://doi.org/10.1007/s00213-010-2010-9

O'Mahony, S. M., Marchesi, J. R., Scully, P., Codling, C., Ceolho, A.-M., Quigley, E. M. M., … Dinan, T. G. (2009). Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biological Psychiatry*, *65*(3), 263–267. https://doi.org/10.1016/j.biopsych.2008.06.026

Paiva, T. O., Almeida, P. R., Ferreira-Santos, F., Vieira, J. B., Silveira, C., Chaves, P. L., … Marques-Teixeira, J. (2016). Similar sound intensity dependence of the N1 and P2 components of the auditory ERP: Averaged and single trial evidence. *Clinical Neurophysiology: Official Journal of the International Federation of Clinical Neurophysiology*, *127*(1), 499–508. https://doi.org/10.1016/j.clinph.2015.06.016

Pisanu, C., & Squassina, A. (2017). We are not alone in our body: insights into the involvement of microbiota in the etiopathogenesis and pharmacology of mental illness. *Current Drug Metabolism*. https://doi.org/10.2174/1389200219666171227204144

Pistollato, F., Sumalla Cano, S., Elio, I., Masias Vergara, M., Giampieri, F., & Battino, M. (2016). Role of gut microbiota and nutrients in amyloid formation and pathogenesis of Alzheimer disease. *Nutrition Reviews*, *74*(10), 624–634. https://doi.org/10.1093/nutrit/nuw023

Radolfova-Krizova, L., Maixnerova, M., & Nemec, A. (2016). Acinetobacter pragensis sp. nov., found in soil and water ecosystems. *International Journal of Systematic and Evolutionary Microbiology*, *66*(10), 3897–3903. https://doi.org/10.1099/ijsem.0.001285

Radzik, I., Miziak, B., Dudka, J., Chrościńska-Krawczyk, M., & Czuczwar, S. J. (2015). Prospects of epileptogenesis prevention. *Pharmacological Reports: PR*, *67*(3), 663–668. https://doi.org/10.1016/j.pharep.2015.01.016

Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews. Immunology*, *9*(5), 313–323. https://doi.org/10.1038/nri2515

Samuel, B. S., Shaito, A., Motoike, T., Rey, F. E., Backhed, F., Manchester, J. K., ⋯ Gordon, J. I. (2008). Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(43), 16767–16772. https://doi.org/10.1073/pnas.0808567105

Sanguinetti, E., Collado, M. C., Marrachelli, V. G., Monleon, D., Selma-Royo, M., Pardo-Tendero, M. M., ⋯ Iozzo, P. (2018). Microbiome-metabolome signatures in mice genetically prone to develop dementia, fed a normal or fatty diet. *Scientific Reports*,

8(1), 4907. https://doi.org/10.1038/s41598-018-23261-1

Scharfman, H. E., Goodman, J. H., Rigoulot, M.-A., Berger, R. E., Walling, S. G., Mercurio, T. C., ⋯ Maclusky, N. J. (2005). Seizure susceptibility in intact and ovariectomized female rats treated with the convulsant pilocarpine. *Experimental Neurology*, *196*(1), 73–86. https://doi.org/10.1016/j.expneurol.2005.07.007

Scharfman, H. E., & MacLusky, N. J. (2006). The influence of gonadal hormones on neuronal excitability, seizures, and epilepsy in the female. *Epilepsia*, *47*(9), 1423–1440. https://doi.org/10.1111/j.1528-1167.2006.00672.x

Scheperjans, F., Aho, V., Pereira, P. A. B., Koskinen, K., Paulin, L., Pekkonen, E., ⋯ Auvinen, P. (2015). Gut microbiota are related to Parkinson's disease and clinical phenotype. *Movement Disorders: Official Journal of the Movement Disorder Society*, *30*(3), 350–358. https://doi.org/10.1002/mds.26069

Sender, R., Fuchs, S., & Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biology*, *14*(8), e1002533. https://doi.org/10.1371/journal.pbio.1002533

Stuart, M. J., & Baune, B. T. (2014). Chemokines and chemokine receptors in mood disorders, schizophrenia, and cognitive impairment: a systematic review of biomarker studies. *Neuroscience and Biobehavioral Reviews*, *42*, 93–115. https://doi.org/10.1016/j.neubiorev.2014.02.001

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.-N., ⋯ Koga, Y. (2004). Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *The Journal of Physiology*, *558*(1), 263–275. https://doi.org/10.1113/jphysiol.2004.063388

Sur, S., & Sinha, V. K. (2009). Event-related potential: An overview. *Industrial Psychiatry*

Journal, *18*(1), 70–73. https://doi.org/10.4103/0972-6748.57865

Švec, P., De Bel, A., Sedláček, I., Petráš, P., Gelbíčová, T., Černohlávková, J., ⋯ Pantuček, R. (2015). Staphylococcus petrasii subsp. pragensis subsp. nov., occurring in human clinical material. *International Journal of Systematic and Evolutionary Microbiology*, *65*(7), 2071–2077. https://doi.org/10.1099/ijs.0.000220

Thompson, C. L., Vier, R., Mikaelyan, A., Wienemann, T., & Brune, A. (2012). "Candidatus Arthromitus" revised: segmented filamentous bacteria in arthropod guts are members of Lachnospiraceae. *Environmental Microbiology*, *14*(6), 1454–1465. https://doi.org/10.1111/j.1462-2920.2012.02731.x

Turski, W. A., Cavalheiro, E. A., Schwarz, M., Czuczwar, S. J., Kleinrok, Z., & Turski, L. (1983). Limbic seizures produced by pilocarpine in rats: behavioural, electroencephalographic and neuropathological study. *Behavioural Brain Research*, *9*(3), 315–335. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/6639740

Uhlhaas, P. J., Pipa, G., Neuenschwander, S., Wibral, M., & Singer, W. (2011). A new look at gamma? High- (>60 Hz) γ -band activity in cortical networks: function, mechanisms and impairment. *Progress in Biophysics and Molecular Biology*, *105*(1-2), 14–28. https://doi.org/10.1016/j.pbiomolbio.2010.10.004

Ullsperger, P., Freude, G., & Erdmann, U. (2001). Auditory probe sensitivity to mental workload changes - an event-related potential study. *International Journal of Psychophysiology: Official Journal of the International Organization of Psychophysiology*, *40*(3), 201–209. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11228347

Vaitkevičius, A., Kaubrys, G., & Audronytė, E. (2015). Distinctive Effect of Donepezil Treatment on P300 and N200 Subcomponents of Auditory Event-Related Evoked

Potentials in Alzheimer Disease Patients. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, *21*, 1920–1927. https://doi.org/10.12659/MSM.894940

van de Wijgert, J. H. H. M., Borgdorff, H., Verhelst, R., Crucitti, T., Francis, S., Verstraelen, H., & Jespers, V. (2014). The vaginal microbiota: what have we learned after a decade of molecular characterization? *PloS One*, *9*(8), e105998. https://doi.org/10.1371/journal.pone.0105998

van Veen, S. Q., Claas, E. C. J., & Kuijper, E. J. (2010). High-Throughput Identification of Bacteria and Yeast by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in Conventional Medical Microbiology Laboratories. *Journal of Clinical Microbiology*, *48*(3), 900–907. https://doi.org/10.1128/JCM.02071-09

Vezzani, A., French, J., Bartfai, T., & Baram, T. Z. (2011). The role of inflammation in epilepsy. *Nature Reviews. Neurology*, *7*(1), 31–40. https://doi.org/10.1038/nrneurol.2010.178

Vezzani, A., Moneta, D., Richichi, C., Aliprandi, M., Burrows, S. J., Ravizza, T., ⋯ De Simoni, M. G. (2002). Functional role of inflammatory cytokines and antiinflammatory molecules in seizures and epileptogenesis. *Epilepsia*, *43 Suppl 5*, 30–35. https://doi.org/10.1046/j.1528-1157.43.s.5.14.x

Wang, L., Christophersen, C. T., Sorich, M. J., Gerber, J. P., Angley, M. T., & Conlon, M. A. (2011). Low Relative Abundances of the Mucolytic Bacterium Akkermansia muciniphila and Bifidobacterium spp. in Feces of Children with Autism. *Applied and Environmental Microbiology*, *77*(18), 6718–6721. https://doi.org/10.1128/AEM.05212-11

Weyrich, L. S., Dixit, S., Farrer, A. G., Cooper, A. J., & Cooper, A. J. (2015). The skin microbiome: Associations between altered microbial communities and disease. *The Australasian Journal of Dermatology*, *56*(4), 268–274.

https://doi.org/10.1111/ajd.12253

WHO | Epilepsy. (2017, February 10). Retrieved January 2, 2018, from http://www.who.int/mediacentre/factsheets/fs999/en/

Wilson, M. T., & Hamilos, D. L. (2014). The nasal and sinus microbiome in health and disease. *Current Allergy and Asthma Reports*, *14*(12), 485. https://doi.org/10.1007/s11882-014-0485-x

Winek, K., Dirnagl, U., & Meisel, A. (2016). The Gut Microbiome as Therapeutic Target in Central Nervous System Diseases: Implications for Stroke. *Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics*, *13*(4), 762–774. https://doi.org/10.1007/s13311-016-0475-x

Winek, K., Engel, O., Koduah, P., Heimesaat, M. M., Fischer, A., Bereswill, S., … Dirnagl, U. (2016). Depletion of Cultivatable Gut Microbiota by Broad-Spectrum Antibiotic Pretreatment Worsens Outcome After Murine Stroke. *Stroke; a Journal of Cerebral Circulation*, *47*(5), 1354–1363. https://doi.org/10.1161/STROKEAHA.115.011800

Witten, L., Bastlund, J. F., Glenthøj, B. Y., Bundgaard, C., Steiniger-Brach, B., Mørk, A., & Oranje, B. (2016). Comparing Pharmacological Modulation of Sensory Gating in Healthy Humans and Rats: The Effects of Reboxetine and Haloperidol. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *41*(2), 638–645. https://doi.org/10.1038/npp.2015.194

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., … Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science*, *334*(6052), 105–108. https://doi.org/10.1126/science.1208344

Xie, G., Zhou, Q., Qiu, C.-Z., Dai, W.-K., Wang, H.-P., Li, Y.-H., … Wang, W.-J. (2017). Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with

refractory epilepsy. *World Journal of Gastroenterology: WJG*, *23*(33), 6164–6171. https://doi.org/10.3748/wjg.v23.i33.6164

Yu, M., Jia, H., Zhou, C., Yang, Y., Zhao, Y., Yang, M., & Zou, Z. (2017). Variations in gut microbiota and fecal metabolic phenotype associated with depression by 16S rRNA gene sequencing and LC/MS-based metabolomics. *Journal of Pharmaceutical and Biomedical Analysis*, *138*, 231–239. https://doi.org/10.1016/j.jpba.2017.02.008

Zhao, Y., Jaber, V., & Lukiw, W. J. (2017). Secretory Products of the Human GI Tract Microbiome and Their Potential Impact on Alzheimer's Disease (AD): Detection of Lipopolysaccharide (LPS) in AD Hippocampus. *Frontiers in Cellular and Infection Microbiology*, *7*, 318. https://doi.org/10.3389/fcimb.2017.00318

Zinöcker, M. K., & Lindseth, I. A. (2018). The Western Diet-Microbiome-Host Interaction and Its Role in Metabolic Disease. *Nutrients*, *10*(3). https://doi.org/10.3390/nu10030365

Zoetendal, E. G., Collier, C. T., Koike, S., Mackie, R. I., & Gaskins, H. R. (2004). Molecular ecological analysis of the gastrointestinal microbiota: a review. *The Journal of Nutrition*, *134*(2), 465–472. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/14747690

Acknowledgments

During the PhD program I had a chance to connect, to meet and to inspire from numerous amazing people. I want to take this opportunity to thank each and every one.

My supervisor Prof. Paolo Fabene, for the opportunity to develop this risky, exciting and (what's emerging) very promising idea. Thank you for the chance to be truly in love with the work that was done, for the doses of inspiration, and for the opportunity to grow up scientifically and personally.

All the collaborators from the University of Verona: Beatrice Salvetti, Federico del Gallo, Sabrina Marra, Cristina Patuzzo, Pietro Bontempi, Elena Nicolato, thank you for the team-spirit, for your time, for your passion, for sharing your priceless experience with me.

I want to express a particular gratitude to the Dr. Grygoriy Tsenov: thank you for your endless support and for guiding me through all the ups and downs. It was a real luck to meet you, to work with you and to learn from you.

Our collaborators from the University of Copenhagen: Prof. Axel Kornerup Hansen and Prof. Dennis Nielsen. Thank you for the invaluable practical advices to establish and perform the experiments in the best possible way.

Team of the University of Copenhagen: Randi Lundberg and Tomasz Maciej Cieplak. Thank you for sharing with me microbiota-related lifehacks, tips and tricks and other priceless "know how" that I would never learn from the books.

To my husband, Dmitry Kosenkov for being my best team, as always.