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Behavioral Effects of Faecal Microbiota Transplantation of Individuals with Bipolar Disorder to Female Mice

Bachelorthesis

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Submitted by

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1. Introduction

For this thesis the behavioral effects of fecal microbiota transplantation (FMT) of individuals with bipolar disorder (BD) to mice was studied. Some psychiatric diseases are proven to be connected to the gut microbiota (GM) however, BD is not one of them yet. These experiments should give an idea whether BD is linked to changes in the GM or not. Therefore, behavioral tests for rodents were performed with mice after transplanting gut bacteria from individuals with BD.

1.1 Bipolar Disorder

BP is a severe mental illness defined by extreme mood changes. There is a range of symptoms, which vary in different episodes. On one side is the manic episode, coming with increased euphoria, activity, and irritation. On the other side is the depressive episode, which is defined in symptoms like depressive feelings, sleeping issues and not being able to do simple tasks. The symptoms are equal to those of major depression and often the cause of misdiagnosis. Another episode to differ from is the mixed one, which has symptoms of both, manic and depressive. For mania there is also a mild form detected, which is called hypomania. In this state the symptoms are not severe and sometimes even family members or close friends cannot detect the episode (Miklowitz and Gitlin 2014, Bipolar Disorder 2019).

As in most psychiatric illnesses, the development of BD consists of various factors. Heritability is a great factor, as are stress and biology (Kasper et al. 2013). In 2017 the total number of reported people with BD worldwide was approximately 46 million, with a tendency to affect women more likely than men. Most commonly the disorder occurs in 20- to 24-year-old young adults, but also children are affected. Even if BD is not the most common mental health disease worldwide, the severity should not be underestimated. Next to the burden in daily life tasks, up to 50 % of the patients are estimated to attempt suicide. There is a higher tendency if BD emerged at a younger age (Latalova et al. 2014, Dattani et al. 2021).

1.1.2 The Types of Bipolar Disorder

In clinical terms, there are three main subtypes of BD and unspecified BD. Every case, which cannot be assigned clearly to a subtype, belongs to the unspecified class.
Bipolar I disorder is determined in at least one manic episode, which can or cannot evolve to depressive episodes. The bipolar II disorder is classified in hypomanic episodes, however not

manic ones, and major depression. If once diagnosed with this form of BD, most do not relapse into symptoms for bipolar I disorder. In both forms the mixed state, an episode with contemporary (hypo–)mania and depression or a so-called rapid cycling of the moods, could occur. In cyclothymic disorder or cyclothymia the symptoms of hypomanic and depressive episodes last at least for two years in adults and are not as severe as in bipolar I or II (Miklowitz and Gitlin 2014, Bipolar Disorder 2019).

1.1.3 Tests and Screenings for Bipolar Disorder

BD comes with a lot of different symptoms and especially for depression and mania there are instruments to evaluate the state of the mental illness (Sajatovic et al. 2015). In the next chapters, four widely used instruments for diagnosis are described. One form is peer-assessment, which consists of a 15 to 30 minutes interview performed by a trained psychiatrist, who uses a questionnaire for guidance and then rates the patient´s severity of the illness. Another form is the self-report, where the patients must rate the symptoms themselves.
Common examples for test with peer-assessment are the Hamilton Depression Scale (HAMD), which is a measurement of depression and Young Mania Rating Scale (YMRS) to report the severity of mania. Self-report tests are for example Beck Depression Inventory (BDI) for depression and Self-Report Manic Inventory (SRMI) for mania.
All the screenings are based on questions, which are connected to symptoms of depression or mania. Based on the answers, points are given and summed up to get a total score, which indicates the severity (Beck et al. 1961, Young et al. 1978, Williams 1988, Bräunig et al. 1996, Sharp 2015).

1.1.4 Treatment for Bipolar Disorder

In addition to the right medication, frequent sessions at a psychotherapist are recommended. This helps to overcome or reduce severe episodes and gives the stability for patients to get back to a normal life (Butler et al. 2018). For medication so far anticonvulsants, antipsychotics, and mood stabilizers are used. Organizations like World Federation of Societies of Biological Psychiatry and International Society for Bipolar Disorders publish guidelines for choosing drugs.
It is important to track the exact episode and subtype of BD for describing the appropriate medication. Even if detected correctly, some patients do not show improvements of symptoms, and a change in treatment is needed. There is not one general medication for each episode or type of BD, because everyone’s body reacts different and in one patient a drug may take effect,

but in another patient leads to aggravation (Nivoli et al. 2012). For identification of the episodes and symptoms, screening instruments as described in the previous chapter are used. However, diagnosis of BD episodes is difficult, especially mixed stages are hard to distinguish (Vieta and Morralla 2010).

All drugs are aimed to stabilize the mood of the patient and treat acute symptoms. For (hypo-)mania and mixed episodes monotherapy with lithium, valproate or atypical antipsychotics is recommended. If medication with one drug alone is not leading to improvement of the patient's mental health, a combination of drugs is suggested. The primary choice for treating mania without depressive symptoms is lithium, while in mixed states valproate and carbamazepine are advocated. Carbamazepine has limitations, because of its interaction with other medications and its fast development of tolerance. Antidepressant therapy should be stopped in all cases of mania. For treating depression, atypical antipsychotics as lurasidone or olanzapine are used (Vieta and Morralla 2010, Bourin and Thibaut 2013).

In general, antipsychotics are widely used for treatment. Both types, typical antipsychotics, also referred to as First Generation Antipsychotic Drugs, and atypical antipsychotics, called Second Generation Antipsychotic Drugs, are used for treating mania. The first ones are rarely used because they are known to increase the risk of extrapyramidal symptom. This is a drug-induced disorder, which is expressed through movement dysfunctions like dystonia and abnormalities also seen in Parkinson´s disease (PD). Additionally to depression treatment, atypical antipsychotics are used for maintenance treatment. In all cases they can be used as monotherapy or mixed with other drugs (Barnes and McPhillips 1998, Janicak et al. 2011).

Despite the good effects these medications have on patients there is a wide range of side effects which should be taken seriously. The most common, and mostly harmless, side effects detected in many of the medicines are dizziness, tiredness, and nausea. To point out some severe effects, lithium could lead to kidney-, thyroid-, intestine- and heart- problems. Lithium has been used for a long time and therefore the side effects are studied extensively. Sometimes it is possible to lower the effects by reducing the dosage. However, knowing the adverse effects is not improving them and every new treatment found with less or ideally no harmful effects results in a benefit for the patients (Bowden 1998, Kasper et al. 2013).

1.2 Gut-Brain Axis

The brain is known as the main organ of being in control of the body. However, in the last decades, the attention was brought to the influence the GI tract has on the brain. This bidirectional interaction is called “gut-brain axis”.

Microorganisms are using signaling molecules to communicate with the brain. The molecules bind to the target, which could be another microbe, cell or even the nervous system. Part of this process are enterochromaffin cells, which transduce information of the GI tract to the nervous system and the other way around (Rhee et al. 2009). They are a subtype of enteroendocrine cells, and are found in the epithelium of the GI tract and therefore on the luminal side close to the microbiota metabolites. On the basolateral side they affect afferent and efferent nerve endings (Bistoletti et al. 2020). Through the wide range of their expressional receptors, these cells are essential for signaling. Especially serotonin-receptors, as serotonin is a neurotransmitter (NT) in the peripheral nervous system. The principal amount of serotonin is produced, stored, and released in enterochromaffin cells. Other subtypes of enteroendocrine cells are responsible for releasing gut peptides as signaling molecules (Hansen and Witte 2008).

1.2.1 Gut Microbiota

The GM is defined as the community of microorganisms in the GI tract. The organisms are mostly viruses, bacteria, and fungi with a tremendous variety. Their composition is influenced by a lot of factors, for example ethnicity, age, or diet. Aside from the influence of the brain, which was discussed in the previous chapter, the microorganisms support the body in a lot of ways. One task is the exploitation of certain non-digestive products. They are fermented to short chain fatty acids (SCFAs), which are used in many metabolic pathways as gluconeogenesis and β–Oxidation (Valdes et al. 2018). Another important function is the maintenance of the immune system. The organisms in the gut compete with pathogens, which prevents infections, and also affect the lymphatic development (Kamada et al. 2013).

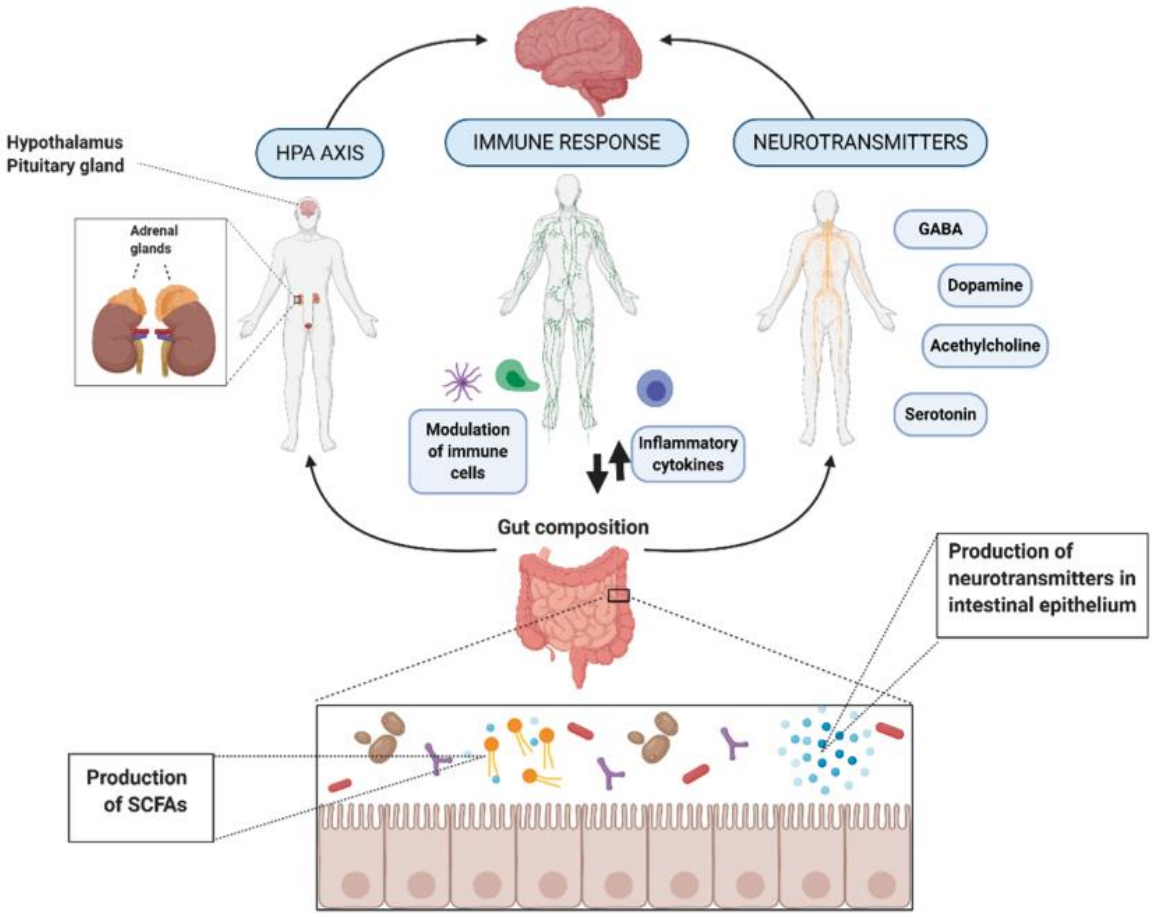

Figure 1: Influence of the GM on the body (Toro-Barbosa et al. 2020)

In Fig. 1, the influence of the gut composition on the body, especially the brain, is shown. The GM effects the production of short chain fatty acids (SCFAs) and neurotransmitter (NT) and they are responsible for pathways in the brain as well as the modulation of the immune system.

1.2.2 Fecal Microbiota Transplantation

As the GM is affected by a variety of diseases and the imbalance itself can cause illnesses, treatments via altering the bacteria in the gut were established. FMT is such a tool. This is a procedure, during which prepared fecal matter from a donor is brought into the recipients gut, with the prospect of changing the GM to a healthy composition (Gupta et al. 2016). To prepare the feces, they are mixed with a saline solution and filtered. Colonoscopy, endoscopy, a nasogastric tube or an enema can be used to place the finished solution inside the patient (Nicco et al. 2020) .

The donor must be screened carefully beforehand, to ensure that no harmful bacteria or pathogens will harm the recipient. To demonstrate the process of donor screening, the evaluation for donors, suitable for FMT in patients with *Clostridium difficile* infections, for a public stool bank is described in the next paragraph.

First, the participants must complete an online registration. After that, potential attendees undertake a clinical assessment, to rule out the risk of transmittable diseases and then a stool screening and a serological screening takes place. The stool testing consists of a few assays for pathogens which are e. g. *C. difficile*, *Salmonella*, *Heliobacter pylori* and *Microsporidia*. For serologic testing HIV antibodies, Hepatitis A, B and C, a screening for hepatic functions, and other tests are executed. At the third and last stage only 2.8 % of the participants were considered qualified as donors (Dubois et al. 2015).

The use of FMT is reported most successful in *C. difficile* infections. This pathogen is a toxin-producing bacillus, often transmitted in hospitals, which leads to diarrhea with a possibility to death (Czepiel et al. 2019). In 2012, Mellow and colleagues achieved a primary cure rate of 91 % and a secondary cure rate of 98 % in patients with recurring *C. difficile* infections. The primary cure rate was achieved if the symptoms of the disease disappeared within 90 days after FMT. The secondary cure rate was defined if no symptoms existed after the additional treatment with vancomycin and in some cases after another FMT. They concluded that for individuals with *C. difficile* infections FMT is a safe and acceptable treatment (Mellow et al. 2012). In general, for safety concerns, FMT is considered a treatment without severe side effects (Borody et al. 2012).

1.2.3 Fecal Microbiota Transplantation in Mental Health Diseases

To show the importance of the brain-gut axis in psychiatric illness, in the last years many studies focused on performing a FMT in rodents with the purpose to show a connection. With autism spectrum disorder (ASD) they even performed FMT on humans. ASD includes a range of neurodevelopmental conditions referring to challenges in social interaction and the need for repetitive behaviors.

In 2019, Sharon and colleagues executed a study, to show the connection between ASD and the GM. They performed FMT of humans with the disease into germ-free mice and observed abnormal behavior (Sharon et al. 2019a). In another study, the GM showed a difference between children with ASD and control groups as well as in ASD mouse models (Coretti et al. 2017, 2019). In addition, FMT and treatments with antibiotics and probiotics have shown an improvement in human trials. In ASD the symptoms did not just improve after FMT with

additional treatments, the improvement remained for two years (Sandler et al. 2000, Grimaldi et al. 2018, Kang et al. 2019).
Other psychiatric diseases, which showed evidence of a connection with the GM are PD and major depression. PD is a neurodegenerative disorder, characterized by motor symptoms like bradykinesia and non-motor symptoms like GI dysfunction (Sun et al. 2018). In this disease the GM is proven to be altered in patients. With this background a FMT of PD mice in healthy mice was performed and revealed that the healthy mice showed symptoms of the disease afterwards. In depression the altered GM is proven as well (Park et al. 2013). Additionally, FMT from depressed patients to rat models was carried out and lead to behavioral and physiological changes (Kelly et al. 2016a).

To show an altered behavior of the rodents after FMT, several experiments were executed in the studies. The most common one was the open field test (OFT), which measures anxiety. Other experiments for anxiety-like behavior were marble burying, step down, sucrose preference, elevated plus maze (EPM) and forced swim test. For depression in PD models the tail suspension test was used. In tests for ASD the sociability was measured with the three-chamber sociability test and the direct social interaction test (Park et al. 2013, Kelly et al. 2016b, Sharon et al. 2019b).

1.3 Anxiety

1.3.1 Mechanisms of Anxiety

The approach of anxiety is important for two reasons: Evidence shows that patients with BD are likely to develop anxiety disorder as well, and secondly the similarity between depression and anxiety in mouse models. If not only BD, but anxiety disorder is present, the symptoms of BD seem to increase (Lee and Dunner 2008). But more important is the aspect of animal models that express BD. In the laboratory it is not yet possible to mimic all aspects of the illness in solely one model. The model is either suited for mania or for depression (Beyer and Freund 2017). Depression and anxiety have similar physical appearances and therefore behavioral tests in animal models could overlap (Paul 1988).

At first the two reactions - anxiety and fear - are seen as one, but even though both have similar physiological reactions, they differ. Fear comes from a certain threat, while anxiety has no immediate threat, but a linked emotion to it. For example, if someone’s house is shaking and

on the verge of breaking down, the emotion would be fear. However, if someone would worry because of a storm outside, but the house is in no danger the experienced feeling is anxiety (Elman and Borsook 2018).

1.3.2 Responsible Brain Regions

There are two pathways in the brain to trigger anxiety. One via the cerebral cortex, which is responsible for processes like consciousness and emotions. Overthinking problems and doubts is triggering anxiety in the cortex. The other pathway is to the amygdala. The amygdala is located on both sides of the brain, inside the temporal lobe. This small structure has lots of connections to other areas, which trigger physical responses like increased blood flow and dilated bronchi to prepare the body for fighting or fleeing. The several sections of the amygdala can be seen in Fig. 2, but for anxiety primarily the lateral nucleus (LA) and the central nucleus (CeA) are responsible. While the cortex processes information by thinking, the amygdala lets the body react before the cortex even is aware of the danger.

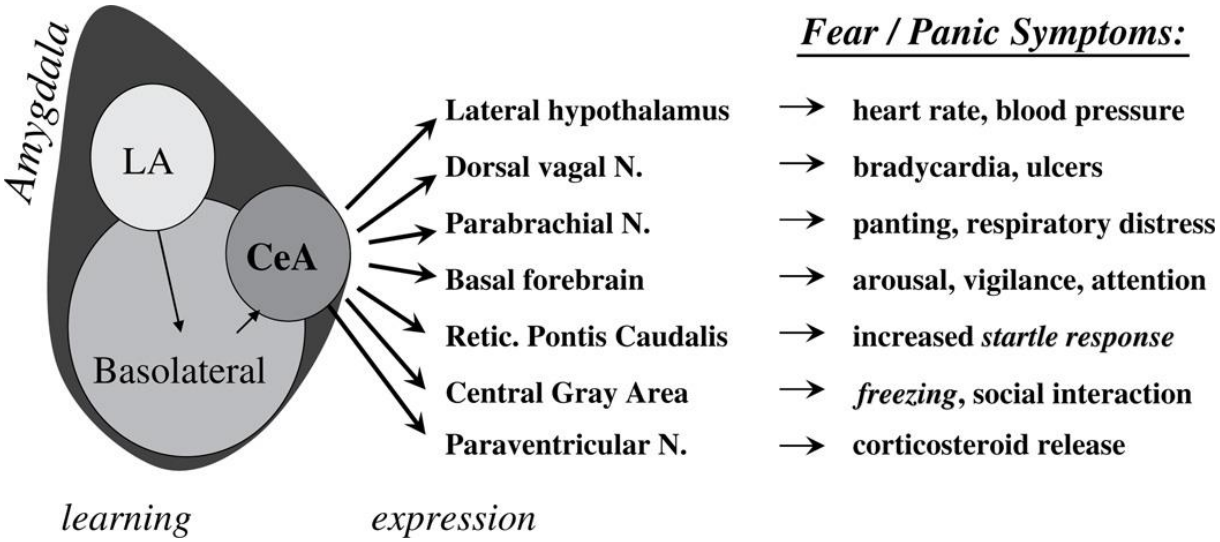

Figure 2: The amygdala and its response to fear (Martin et al. 2009)

This figure shows the lateral nucleus (LA), the basolateral nucleus and the central nucleus (CeA) of the amygdala. The CeA transfers information to all the brain regions listed, which trigger the measurable fear and panic symptoms.

To get information to the cortex, the sensory organs are responsible to catch sensations from the environment. These will then be sent to the thalamus, where the transfer to the responsible

region for processing takes place and after that the signal travels to the amygdala, which induces the response of the body.
For the faster pathway to the amygdala, the LA is responsible for receiving information of the sense organs and the CeA passes the signal on to the sympathetic nervous system and the hypothalamus. The sympathetic nervous system eventually leads to fight, flight or freeze reactions in the body. In the hypothalamus, the hormones cortisol and adrenaline are mediated and trigger a response of the body (Pittman and Karle 2015).

Brain imaging, especially magnetic resonance imaging has a major potential in detecting abnormalities in the brain. So far it is not fully understood how BD affects the brain, but there is some evidence.
Phillips and Swarts summed up the findings from neuroimaging studies which showed abnormalities of patients with BD (Phillips and Swartz 2014). The first alteration is in the regions for emotion processing and regulation, of which the amygdala and different prefrontal cortical regions are part of. This was pictured with an increased amygdala activity and a decreased activity of the regions in the prefrontal cortex (Phillips et al. 2008). If exposed to positive stimuli and cognitive tasks, these regions seem to be abnormal in patients with BD (Lawrence et al. 2004, Cremaschi et al. 2013). Additionally, the reward center is more sensitive to reward than in healthy individuals (Alloy et al. 2012).
Moreover, a decreased volume in grey and white matter in BD patients as in their relatives was detected (Matsuo et al. 2012). Connecting to the emotional findings, the amygdala and hippocampus have a reduced volume during depressive episodes (Wijeratne et al. 2013).
Postmortem a reduction of neuronal and glial density in BD patients in the dorsolateral prefrontal cortex was found (Rajkowska et al. 2001). In contrast, another study could not reproduce those findings, but a decrease in neuronal sizes was found (Cotter et al. 2002).

1.3.3 Responsible Neurotransmitters

There are many NT involved with the response to anxiety. Corticotropin releasing factor (CRF) is released from projecting neurons and mediates the body's response to stress. If it encounters the amygdala, CRF is known for its anxiogenic effects. Glucocorticoids, especially corticosterone in rodents, leads to anxiogenic and dendritic hypertrophy in the amygdala. Noradrenaline is activated in the stress associated regions of the limbic system and leads to anxiety. The NT serotonin is known for its anxiolytic effects. Therefore, serotonin shortage could cause diseases like depression (Baldwin and Rudge 1995). The gamma–aminobutyric

acid (GABA) system is known for its anxiolytic effects, with its inhibitory effect on the central nervous system. On the opposite site there is glutamate, which has exhibitory effects and increased levels of this NT lead to anxiety (Kim and Gorman 2005).

For integrity, the following mentioned NT are also known in correlation with anxiety. Acetylcholine and cholecystokinin are known for their anxiolytic effects, while substance P is known for increased anxiety. However, the network of NT is very complex and if one transmitter influences another or binds to an altered receptor, most of the transmitter can influence anxiety in positive and negative ways, which is often associated with anxiety disorders (Kaur and Singh 2017).

1.3.4 Influence of the Gastrointestinal tract

For anxiety-like behavior controlled by the GI tract three main types of peptides are considered as important: Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) (Lach et al. 2018). All these peptides are linked to the gut-brain axis. PYY is released in the lower GI tract, where it leads to a reduction of the stomach mobility. PP is released after a meal in the pancreas to reduce pancreatic secretion. Both peptides then lead to satiety. Other than PYY and PP, which are primarily expressed by the digestive system, NPY is released in every stage of the axis including the brain. In the gut the main place of release are enteric neurons (Holzer et al. 2012, Reichmann and Holzer 2016). The meaning of NPY in stress situations is described with anxiolytic effects. If an individual is exposed to chronic stress, NPY has been shown to link with the involvement of depression in animal models (Heilig 2004). All the peptides play an important role in maintaining the energy homeostasis. If disturbed, this could lead to inflammation which later on increases the risk of mood disorders (Holzer et al. 2012).

As it was previously discussed, the composition of the bacteria in the gut, influences many mechanisms and diseases, it is obvious that some bacteria also might influence anxiety. One is the probiotic *Lactobacillus plantarum P8*, where a study showed improvement in stress, cognitive skills and anxiety of stressed adults (Lew et al. 2019). In general, there is a group of probiotics called psychobiotics, which are living organisms with a beneficial impact on the psychical health. They function through the influence on the hypothalamic-pituitary-adrenal (HPA) axis, the immune system and NT. The HPA axis is important, because it is responsible for stress response in the body. The immune system is regulated by the GM via immune cells and inflammatory cytokines, which are overproduced in a disturbed host-microbiota ratio. The

most strains with psychobiotic potential have been seen in the genus of *Lactobacillus* and *Bifidobacterium* (de Araújo and de Paulo Farias 2020, Toro-Barbosa et al. 2020).

2. Aim of the Study

With the previous introduction, the important role of the GM to the brain was pointed out. As experiments with the connection to BD are limited, this thesis presents our findings on the impact of FMT in behavior in mice.

To achieve our aim, we performed three common behavioral test where we measured several parameters to check, if the experimental group showed anxiety-like behavior.

Following questions are answered through the thesis:

- Were the mice with a FMT of the person with BD more anxious in the tests than the control group with the FMT of a healthy person?
- In which behavioral tests was a difference detected between the two groups of mice?
- What do the results of the experiments mean for the future of individuals with BD?

3. Material and Methods

3.1 Material

3.1.1 Experimental Animals

The ethical committee at the Federal Ministry of Education, Science and Research of the Republic of Austria approved the performed animal experiments (permit BMBWF–66.010/0047–V/3b/2019). For ethical reasons the number of animals was limited, and their suffering was minimized.

The used animals were female C57Bl/6J mice, which were bred at the Division of Pharmacology of the Medical University of Graz. Their age varied between 31 and 37 weeks.

3.1.2 Experimental Groups

Based on age and weight the mice were divided in an experimental group and a control group, each with seven animals. The fecal sample of the bipolar group was collected from a female patient with mixed form BD, while the sample for the control group came from a healthy woman. The BD patient was screened and tested for BD beforehand, with test results that are significant for a mixed episode, as showed in Tab. 1.

Test	Value	Meaning
BDI	47	Severe depression
HAMD	20	Moderate depression
SRMI	37	Manic symptoms
YMRS	14	Slightly manic

Table 1: Screening results of the female patient with diagnosed BD

3.1.3 Chemicals

Aqua bidest. (Fresenius Kabi Austria GmbH, Austria)

Ethylenglykol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Tri-Natriumcitrat-Dihydrat (Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

3.1.4 Equipment

Camera (Basler AG, Germany)

Eppendorf Tubes (Greiner, Austria)

Pipette tips (Greiner, Austria)
Pipettes (Gilson, Austria)
Scale (Kern 575, Kern & Sohn GmbH, Germany)

3.2 Methods

3.2.1 Antibiotic Treatment

To minimize the inherent gut-microbiota, the mice were treated with a validated mixture of antibiotics. The mixture consisted of Meropenem (1 mg/ml for 7 days via drinking water, AstraZeneca Austria GmbH, Vienna, Austria), Neomycin (5 mg/ml for 7 days via drinking water, Sigma-Aldrich Handels GmbH, Vienna, Austria) and Vancomycin (0,3 mg/ml for 7 days via drinking water, Sigma-Aldrich Handels GmbH, Vienna, Austria). The animals were weighed before and after administration. After the treatment they were given the usual tap water and no food until the FMT 24 hours later.

3.2.2 Fecal Microbiota Transplantation

The FMT was carried out via intracolonic infusion. The infusion was performed with a catheter, inserted about 3 cm into the anus. To spare the animals, before FMT every mouse was sedated with Isoflurane (3–5 l/min, Rothacher-Medical GmbH, Berne, Switzerland) via inhalation. The injection into the colon contained about 200 µl of the fecal samples.

3.2.3 Open Field Test

The OFT is a method to measure the anxiety-like behavior of laboratory animals. The animals are, one by one, contained in a squared box surrounded with walls for a total time of five minutes while their behavior is analyzed. Evolutionary, rodents tend to avoid unknown open areas, which is tracked in the OFT. Related to this behavior the first exposure to the open field can show anxiety and eventual changes in their performance due to experimental interventions, for example drugs (Choleris et al. 2001). For this test, the instinct of mice remaining to close walls is assessed. Naturally, this decreases the longer the mice are exposed to the field. With anxiogenic drugs this behavior is reduced, which leads to the statement that rodents with anxiety tend to avoid the open field even more than healthy ones (Simon et al. 1994).

Most of the models of the boxes used in studies are similar. In this study a box with walls out of opaque grey plastic was used. The dimensions of the outer edge were 50 x 50 cm and of the center zone were 36 x 36 cm (Fig. 3).

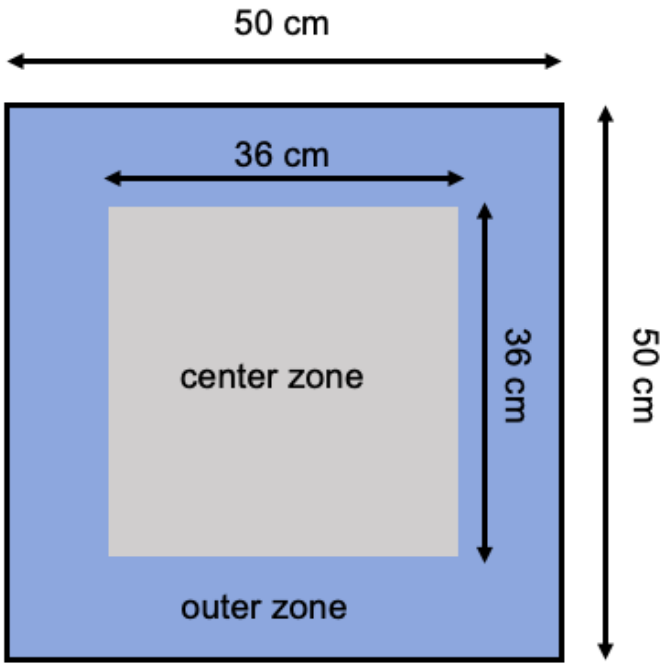

Figure 3: Schematic representation of the OFT

The placement of the outer zone is demonstrated in blue with the measurements 50 x 50 cm and the center zone is illustrated in grey with 36 x36 cm.

To analyze the animals' movements an overhead camera recorded them, and they were tracked with a special software (EthoVision XT 12, Nodulus Information Technology, the Netherlands). To increase the reliability of the test all the animals were released from the same corner and the measurement started immediately. After every trial, the fecal boli were counted and collected, as well as the box was cleaned with water and ethanol.

3.2.4 Elevated Plus Maze

The EPM is a test for checking the anxiety-like behavior. For this method a construct with two open and two closed arms, connected in the center area, is used. The closed arms are enclosed by walls, while the open arms remain without walls (Komada et al. 2008). The

purpose of this test is again to study the inherent behavior of avoiding unknown open areas (Choleris et al. 2001, Bailey and Crawley 2009).

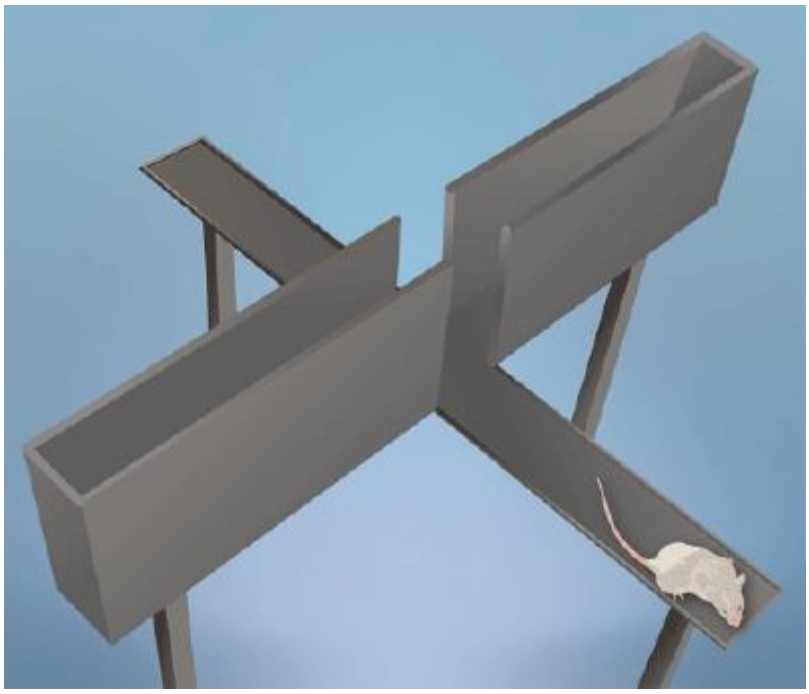

Figure 4: Schematic picture of the EPM (E-Phy-Science 2019)**.**

This illustration is a schematic representation of the two types of arms in an EPM. They are arranged in a cross form, with the middle referred as center point.

With an overhead camera and the visualizing software (EthoVision XT 12, Nodulus Information Technology, the Netherlands) the number of entries and the time spent in the open arms was analyzed. After every trial, the fecal boli were counted and collected, as well as the box was cleaned with water and ethanol.

3.2.5 Light/Dark Box Test

The light/dark box test (LDT) is a test to measure anxiety-like behavior. In this experiment, the natural behavior of rodents to avoid bright areas, is used for analytics. A box with two equal sized chambers, a dark one and a bright one, is provided. The laboratory animals are allowed to move through a small hole in the middle. As the bright light leads to stress, animals with higher anxiety levels are most likely to remain in the dark chamber (Takao and Miyakawa 2006a).

The test was performed by releasing the mice in between the chambers with their head pointed in direction of the dark box. Beforehand the lamps pointing to the bright chamber were calibrated to 280 Lux. With a software (LabMaster, Accurate Info Solution, India) the time spent in the light box and dark box was measured for a duration of ten minutes. An analysis of the distance moved, and rearing was carried out as well. It is called rearing, if a mouse is standing on their hind legs.
After every trial, the fecal boli were counted and collected, as well as the chambers were cleaned with water and ethanol.

3.2.6 Statistics

All the statistical tests performed on the data were executed with GraphPad Prism (Version 9.1.2 (225)). A basic analysis to evaluate the minimum, maximum, mean, and standard error of mean (SEM) was done. Beforehand a Shapiro-Wilk test and a Kolmogorov-Smirnov test were applied on all datasets to check the normal distribution. After that an unpaired t-test was performed to check the significance of the difference between the groups. The threshold for the p-value was set at 0.05, which defined all results with a p-value lower than this as significant.

4. Results

4.1 Open Field Test

The measured parameters were examined and pictured using GraphPad Prism (Version 9.1.2 (225)). An unpaired t-test was performed to check the significance of the difference between the groups.

For the OFT five different parameters were investigated. First, the latency of going into the center zone (Fig. 5). Second, the counts going in the center zone (Fig. 6), as well as the cumulative duration in the center zone (Fig. 7). For locomotion, the distance moving (Fig. 8) and the time not moving (Fig. 9) was measured.

We did not find a significant difference between the bipolar and the control group. However, a tendency of the control group to go more often into the center zone and of the experimental group not to move was distinguishable.

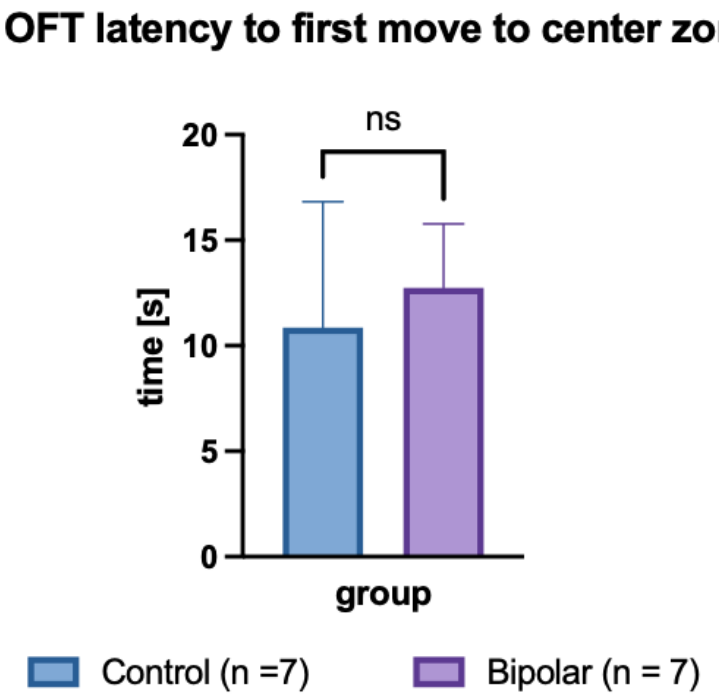

Figure 5: Latency to first move to the center zone in the OFT shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

The measurement taken in Fig. 5 was the latency time of the mice move into the center zone. With a p-value of 0.78 the difference between the groups was not significant. The mean of the

control group was at 10 seconds and the experimental group took the first step on average after 13 seconds, which indicate a sparse tendency of the bipolar mice to move first.

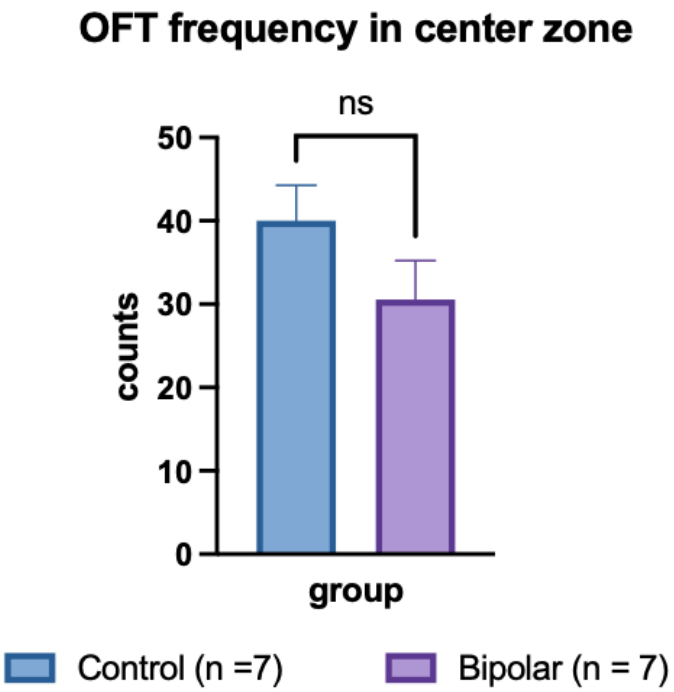

Figure 6: Frequency in the center zone of OFT shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

Fig. 6 shows how often the mice entered the center zone of the OFT within the measured time of five minutes. While the animals belonging to the control group entered the center zone on average 40 times, the bipolar group´s mean was at 31 times. With a p-value of 0.16, the difference between the groups was pointed out not significant.

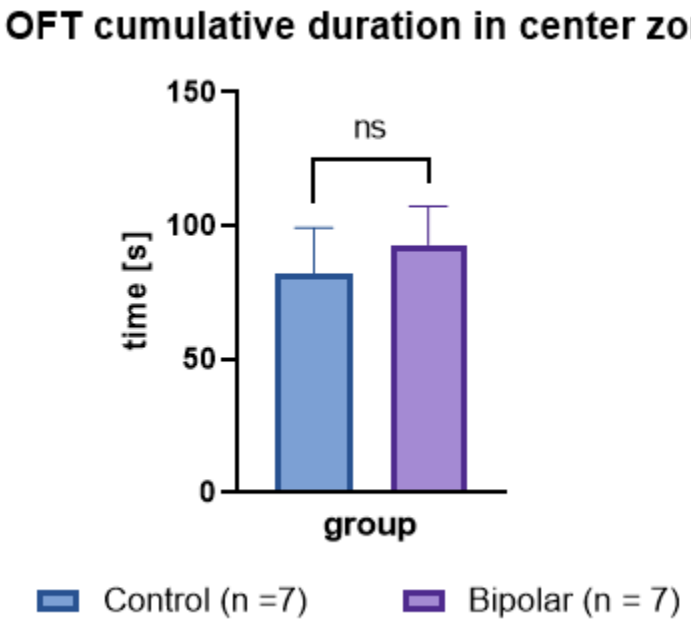

Figure 7: Cumulative duration in the center zone of the OFT shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

Here it is illustrated how long the mice stayed in the center zone. With a p-value of 0.63, the difference between the groups was pointed out not significant. The means differed 11 seconds between the groups, with a slight tendency of the bipolar mice to stay longer in the center. The control group lasted on average 82 seconds and the bipolar group 92 seconds.

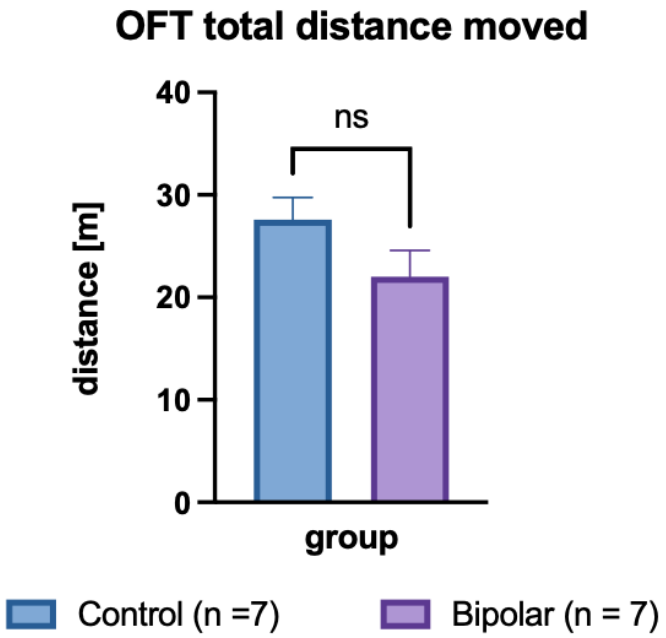

Figure 8: Total distance moved in the OFT shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

Fig. 8 provides information about the total distance moved during the five minutes of the OFT. The control group traveled on average 27.6 m and the bipolar group 22 m. The calculated p-value was 0.12, which does not point out a significance, although the control group tend to move more.

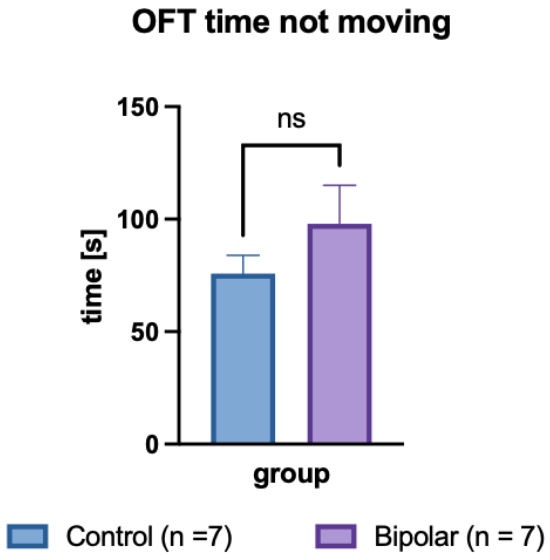

Figure 9: Time not moving in the OFT shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

Fig. 9 visualizes the duration of the mice not moving. With an average of 76 seconds not moving during the whole test, the control group was more in motion than the bipolar group, which stayed still for an average of 98 seconds. However, the p-value of 0.27 indicates no significance.

4.2 Elevated Plus Maze

The software (EthoVision XT 12, Nodulus Information Technology, the Netherlands) measured the movements of the mice, which were illustrated in figures using GraphPad Prism (Version 9.1.2 (225)). For this test, the software measured three points of the mouse: the head, the body, and the tail. In the results only the parameters, where all the points applied were measured.

For the EPM the same parameters, which are the latency time to first move into the open arms (Fig. 10), the duration in open arms (Fig. 11), the number of entries in the open arms (Fig. 12), the total distance moved (Fig. 13) and the cumulative duration in the center point (Fig. 14), were analyzed.

The control group was more likely to go and stay in the open arms than the bipolar group. Additionally, the control was slightly faster to take their first move into the open arms and covered more distance. In the duration in the center point nearly no difference was detected.

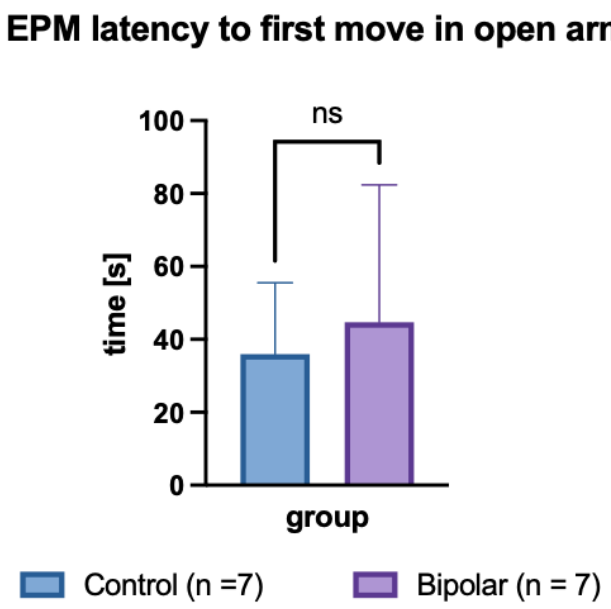

Figure 10: Latency to first move in open arms of the EPM shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

Fig. 10 shows how much time the experimental animal took to move in the open arms of the maze. With the mean of 36 seconds in the control group and 45 seconds in the bipolar group, the control was slightly earlier to discover the open arms. Anyhow, the p-value of 0.8 confirms no significance.

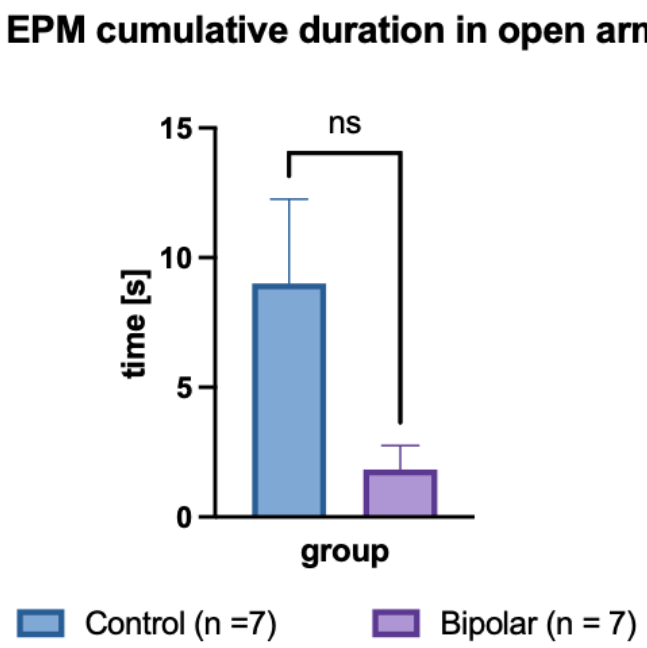

Figure 11: Cumulative duration in open arms shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

The control group stayed in the open arms on average for 9 seconds and the experimental group for 2 seconds. With a p-value of 0.05 the difference is considered statistically not significant; however, the control group was more likely to move into the open arms of the EPM (Fig. 11).

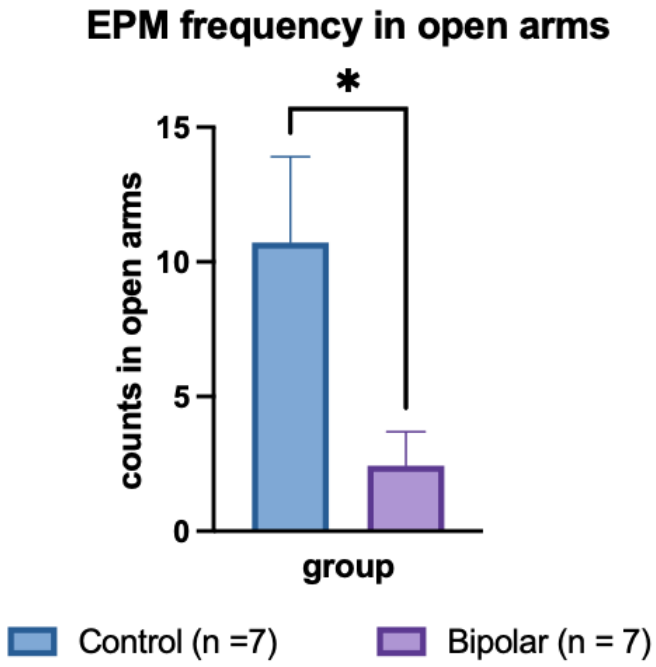

Figure 12: Frequency in open arms shown with the means ± SEM, significance measured with an unpaired t-test (significant with $p<0.05$)

Fig. 12 shows how many times the control and the experimental group entered the open arms of the EPM with the nose, body, and tail. With the control group entering the opens arms on average 11 times and the experimental group twice, the measured p-value was 0.03, which significantly showed the control group being more often inside the open arms.

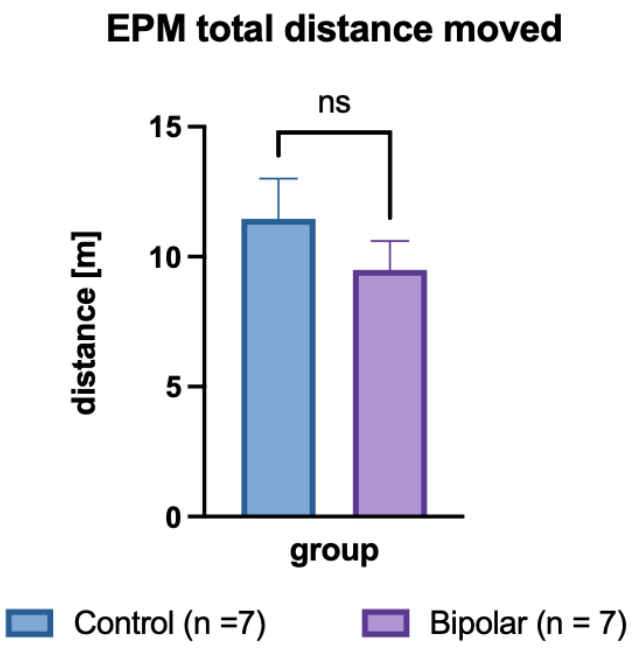

Figure 13: Distance moved in EPM shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

The total distance moved in the 5 minutes of the test is shown in Fig. 13. With a p-value of 0.32, there is no significance. On average the control group moved more, with a mean of 11.5 m, than the bipolar group, which moved about 9.5 m in total.

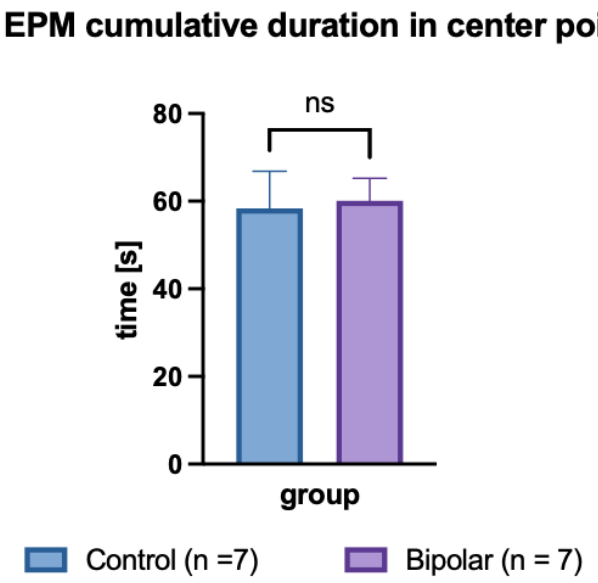

Figure 14: Cumulative duration in center point shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

With the mean of 58 seconds for the control group, 60 seconds for the bipolar group and the p-value of 0.86, there is no significant difference between groups for staying in the center point.

4.3 Light/Dark Box Test

The software (LabMaster, Accurate Info Solution, India) measured the parameters of choice, which were showed using GraphPad Prism (Version 9.1.2 (225)).

For the LTD, the parameters for distance (Fig. 15), time in light (Fig. 16), speed (Fig. 17) and rearing (Fig. 18) were measured. The bipolar group seemed to stay longer and cover more distance in the light box than the control group. On the other hand, they moved faster in the light compartment. In the counts of rearing, no strong difference was shown, but the duration of the animals rearing, the bipolar animals significantly reared longer in the light than the control.

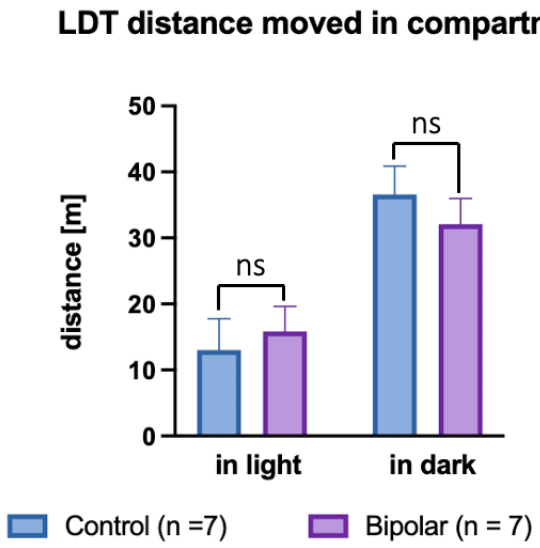

Figure 15: Comparison of the distance in the light and dark compartment shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

In Fig. 15 the difference of the traveled distance between the light and dark compartment and the two groups is shown. It is distinguishable that both groups tend to move more in the dark than in the light. However, the bipolar mice were, with the mean distance of 16 m, slightly more in the light than the control with 13 m. Thus, the control group moved with the average of 37 m more in the dark than the bipolar group with 32 m. In both compartments, the p-value showed no significance, with 0.65 in the light and 0.45 in the dark compartment.

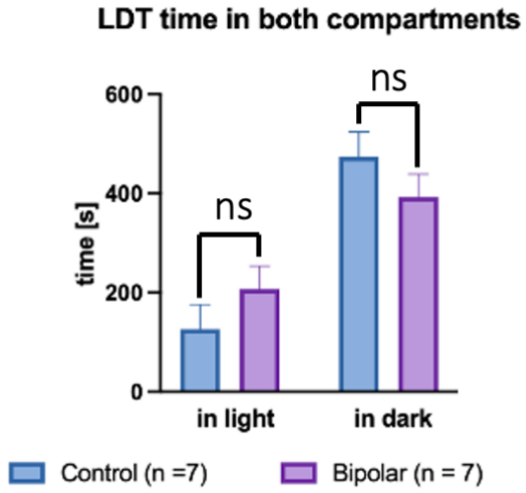

Figure 16: Time spent in the compartments shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

The control group spent more time in the dark than in the light, which is similar to the bipolar group. However, the bipolar mice stayed longer in the light compartment than the control (Fig. 16). The p-value for the difference in the light compared to the dark is 0.25.

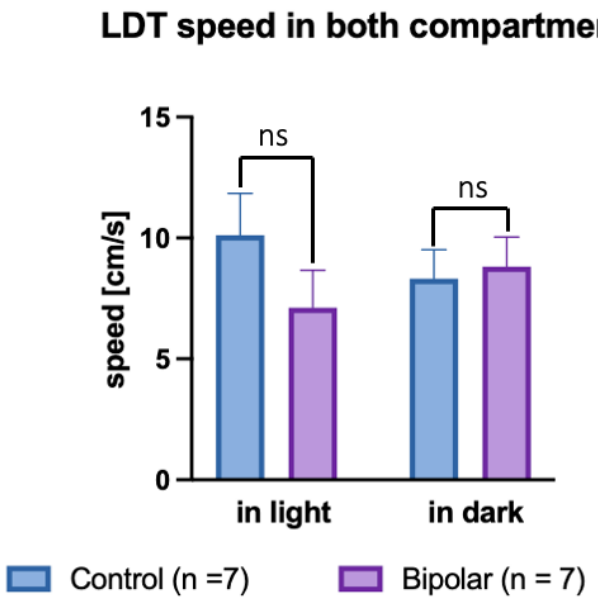

Figure 17: Comparison of the speed in the LDT shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05)

Fig. 17 illustrates the difference between the speed, of the mice in the two compartments. In the light compartment, the p-value was 0.22, with the average speed of 10 cm/s in the control group and 7 cm/s in the bipolar, the experimental group moved slightly faster. In the dark compartment, the p-value is higher with a score of 0.78, because the control group was moving with an average speed of 8 cm/s, nearly as fast as the bipolar group with 9 cm/s. In both cases, the p-value indicates no significance.

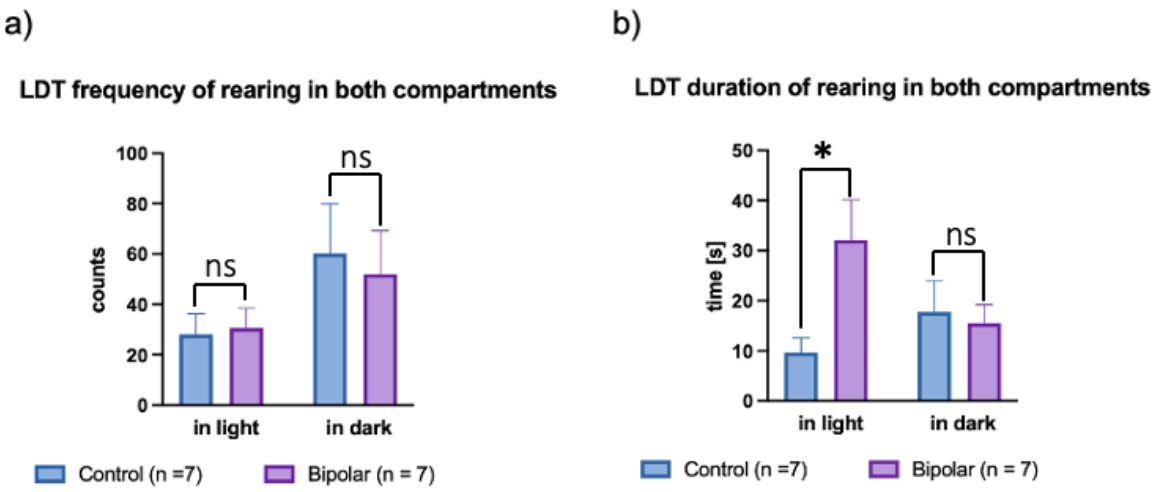

Figure 18: Comparison of the duration and frequency of the rearing shown with the means ± SEM, significance measured with an unpaired t-test (ns with p>0.05, significant with p<0.05)

Fig. 18 shows the duration and frequency of the rearing in the light and dark compartment. Graph a) points out the counts, which were in the control group in the light on average 28 times and in the dark 60 times. The experimental group had a mean of 31 counts of rearing in light and 52 counts in the dark. Both counts show no significance with a p-value of 0.82 in light and 0.76 in the dark compartment. In figure b) is total time of the rearing in both compartments shown. The time of the control group added up to an average of 9 seconds in the light and 18 seconds in the dark. The average of the bipolar mice was at 32 seconds in the light and at 15 seconds in the dark. The difference of the rearing time in light showed a significance with a p-value of 0.02, while in the dark there was no significance detected with p-value of 0.76.

5. Discussion

The results showed an anxiety-like behavior of the experimental group in the EPM, however not in the LDT. Takao and Miyakawa hypothesized that different protocols in laboratories could lead to different results. For example a different brightness of the light in the LDT could affect the outcome. Another hypothesis which would explain our results, is the application of the LDT and the EPM to different impulses. While the LDT tests for bright-space anxiety, the EPM and the OFT detect the open-space like anxiety (Takao and Miyakawa 2006b).
To study anxiety-like behavior and the causalities, many studies were performed. Sziray and colleagues tested the influence of noradrenaline and serotonin. They detected a correlation, even though a contrariety between the results in the LDT and EPM was found. The findings implicate that with lesions in noradrenergic or serotonergic pathways a reduction in anxiety of open space is present. Lesions of both pathways additionally found a reduced bright-space anxiety. This is based on the regulatory impact of anxiety-related processes of these two NT (Sziray et al. 2010).

Another source of the differences could be the testing on different days. The emotional state of rodents is not always the same and therefore leading on some days to more anxiety-like behavior than on others. To minimize the variation of the emotional condition, a triple test, which includes the OFT, EPM and LDT, could be executed. In a single trial all aspects are assessed at once, which limits sources of error occurring through testing on different days, the influence of the test previously performed and the handling between tests (Ramos et al. 2008).

In this project was shown, that a FMT of a patient with the disease into mice, affect their behavior in the EPM. Additionally, in a study alteration in the GM of BD patients and healthy humans was detected. The composition of the bacteria was found to be significantly different as well as a loss in diversity. They are giving an perspective on diagnosing the type and severity via the GM (Hu et al. 2019).

The outlook on future projects would be research of the alteration of the brain and the GM in individuals with BD. With previous research, differences of the sick and healthy were shown, but how these two regions of the body work together in sickness is still not understood.

6. Conclusion

Following questions were answered in this thesis:

Were the mice with a FMT of the person with BD more anxious in the tests than the control group with the FMT of a healthy person? And in which behavioral tests was a difference detected between the two groups of mice?
In the OFT there was a tendency of the bipolar group to show more anxiety-like behavior in the open space detected. Similar result could be found in the EPM, where the control group showed more courage in the open arms. The results in the LDT differed from the other tests, because the experimental group was more likely to stay in the bright area than the control group.

What do the results of the experiments mean for the future of individuals with BD?
In summary, we found anxiety-like behavior of the bipolar mice in two of the three tests, which let us conclude that FMT worked. With this finding future projects could work on understanding the gut-brain axis and therefore providing a treatment via FMT for patients with BD.

7. Summary

Bipolar Disorder (BD) is a mental illness that is defined in extreme mood swings, called mania and depression. While in mania, there is an increased mood and activity, in depression the patients have emotional lows and commonly suicidal thoughts. These mood swings are an extreme burden for the suffering patient, along with relatives and friends.
To find a cure, further research in the connection between the gut and the brain has to be done. One attempt is the fecal microbiota transplantation (FMT), which is the introduction of purified feces from one individual into another. A changed composition of the gut microbiota (GM) alone could lead to diseases or in the case of FMT to a cure. With *Clostridium difficile* infections this has been proven as a successful therapy. However, in mental health disorders there have not been such results due to the lack of research. It has already been shown that the alteration of the microbiome is connected to certain mental illnesses. In Parkinson's disease, major depression, developmental disorders, such as autism, and BD, there are results indicating that the composition of gut bacteria changes. Nevertheless, studies on FMT to cure humans with mental illnesses have not been executed to this day.

The aim of the presented study was to show the link between gut microbiota and bipolar disorder using FMT from a patient with the disorder into female C57Bl/6J mice. The hypothesis was that, if BD is linked to the GM, the experimental animals will show abnormal behavior in behavioral tests after the transfer. Especially an anxiety-like behavior would be expected.
To see a difference in behavior, there was an experimental group that was given the stool sample of a female patient with bipolar disorder and a control group that was given the sample of a healthy female. After that, common experiments to show anxiety-like behavior in the bipolar mice were executed. The used tests were elevated plus maze (EPM) and open field test (OFT), which show anxiety to open areas and light/dark test (LDT), which show anxiety to bright areas. The results of the study showed an anxiety-like behavior of the experimental group in the EPM and OFT, however not in the LDT. This could be explained because of the different applications of the tests.

In conclusion, there is a tendency for increased anxiety in the mice with FMT from the bipolar disorder patient. This supports the hypothesis that BD is influenced by the microbiome. Next steps of research will be to understand how exactly the brain and the GM are connected and influence each other in the mental disease

8. Zusammenfassung

Bipolare Störung ist eine psychische Erkrankung, die sich in extremen Stimmungsschwankungen zwischen Manie und Depression äußert. Während bei der Manie eine erhöhte Stimmung und Aktivität zu beobachten sind, kommt es bei der Depression zu Antriebslosigkeit und häufig zu Selbstmordgedanken. Diese Stimmungsschwankungen sind für den Erkrankten, aber auch für Angehörige und Freunde eine extreme Belastung.
Um eine Heilung finden zu können, sollte die Verbindung zwischen Darm und Gehirn noch weiter erforscht werden. Ein Ansatz für eine Behandlung ist der fäkaler Mikrobiomtransfer (FMT), bei dem aufgereinigte Fäkalien von einer Person in den Darm einer anderen gebracht werden. Das Darmmikrobiom hat einen so starken Einfluss auf den Körper, dass allein eine veränderte Zusammensetzung zu Krankheiten oder, im Falle der FMT, zu einer Heilung von Krankheiten führen kann. Bei Patienten mit *Clostridium difficile* Infektionen hat sich die FMT bereits als erfolgreiche Therapie mit wenig Nebenwirkungen erwiesen. Im Zusammenhang einigen psychischen Erkrankungen wurde bereits erwiesen, dass die Veränderung des Mikrobioms eine Rolle spielt. Bei Parkinson, Depressionen, Entwicklungsstörungen, wie Autismus, und bipolarer Störung gibt es bereits Ergebnisse, die zeigen, dass die Zusammensetzung der Darmbakterien von der in gesunden Menschen abweicht. Jedoch ist eine Behandlung mit FMT noch nicht vollständig ausgereift.

Ziel der präsentierten Studie war es, den Zusammenhang zwischen Darmmikrobiota und bipolarer Störung aufzuzeigen, indem mit dem aufgereinigten Stuhl einer Patientin mit dieser Krankheit eine FMT in weibliche C57Bl/6J-Mäuse durchgeführt wurde. Die Hypothese war, dass die Versuchstiere nach dem Transfer ein abnormales Verhalten in Verhaltenstests zeigen würden, wenn bipolare Störung mit der GM zusammenhängt. Im Falle eines Zusammenhangs wäre ein ängstliches Verhalten der experimentellen Gruppe zu erwarten.
Um einen Unterschied im Verhalten festzustellen, wurde einer Versuchsgruppe die Stuhlprobe eines weiblichen Patienten mit bipolarer Störung und einer Kontrollgruppe die Stuhlprobe einer gesunden Frau verabreicht. Anschließend wurden Verhaltensexperimente durchgeführt, damit ein verändertes Verhalten erkannt werden kann. Dies wurde mit dem häufig verwendeten „open field test“ (OFT), dem „elevated pluz maze“ (EPM) und dem „light/dark test“ (LDT) erreicht. Alle diese Experimente zielen auf das natürliche Verhalten von Mäusen ab offene Areale, beziehungsweise im Fall des LDT sehr helle Flächen, zu meiden. Die Ergebnisse zeigten ein ängstliches Verhalten der Versuchsgruppe im EPM und im OFT, jedoch nicht im

LDT. Dies könnte darauf zurückzuführen sein, dass die Tests auf unterschiedliche Verhaltensaspekte der Mäuse anknüpfen.

Zusammenfassend lässt sich sagen, dass bei Mäusen, bei denen die FMT von der Patientin mit bipolarer Störung durchgeführt wurde, eine erhöhte Tendenz zu ängstlichem Verhalten besteht. Daraus lässt sich ein Zusammenhang zwischen dem Mikrobiom und der bipolaren Störung ableiten. In weiteren Projekten sollte die genaue Verbindung zwischen Gehirn und Darm erforscht werden, um zu verstehen, wie man die Krankheit am besten behandeln könnte.

9. Appendix

9.1 List of Abbreviations

ASD = autism spectrum disorder

BD = bipolar disorder

BDI = Beck Depression Inventory

CeA = central nucleus

CRF = Corticotropin releasing factor

EPM = elevated plus maze

FMT = fecal microbiota transplantation

GABA = gamma-aminobutyric acid

GI = gastrointestinal

GM = gut microbiota

HAMD = Hamilton Depression Scale

HPA = hypothalamic-pituitary-adrenal

LA = lateral nucleus

LDT = light/dark box test

NPY = Neuropeptide Y

NT = neurotransmitter

OFT = open field test

PD = Parkinson´s disease

PP = pancreatic polypeptide

PYY = peptide YY

SCFAs = short chain fatty acids

SRMI = Self-Report Manic Inventory

YMRS = Young Mania Rating Scale

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9.3 List of Figures

9.4 List of Tables