Elucidating the role of the anorectic gut microbiome in value-based decision making and reward signaling

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Abstract

Anorexia Nervosa (AN) is one of the most serious chronic disorders of youth and current treatment strategies are only moderately effective. The physiology at heart of AN remains to be deciphered. Aberrances in value-based decision making, including cognitive inflexibility and excessive self-control, contribute to treatment resistance in AN. Furthermore, the mesolimbic pathway, that plays a prominent role in reinforcement behaviors including value-based decision making, seems to function aberrantly in AN. Gut microbiota affect neurotransmission, mood and cognitive function via the gut microbiota-brain axis and the composition of gut microbiome is altered in AN. Here, we hypothesized that AN-specific dysbiosis is causally linked to putative changes in flexible value-based decision making, behavioral inhibition and ventral tegmental area (VTA) reward signaling during AN development. Thirty-nine recipient tyrosine hydroxylase (TH)-cre rats, surgically injected with cre-dependent GCaMP8s and implanted with optic fibers above the VTA, were pretreated with antibiotics and thereafter reconstituted with the gut microbiota of three patients with restricting-type AN and three healthy controls. Antibiotics- and microbiota-induced changes in cognitive flexibility, behavioral inhibition and VTA dopamine (DA) neuronal responses to reward, reinforced and non-reinforced lever presses were examined using the probabilistic reversal learning task, Go/NoGo task and concomitant fiber photometry. Both antibiotics-induced and AN-specific dysbiosis were found to be insufficient to induce changes in reversal learning performance and VTA DA neuronal responses to sucrose reward and reward prediction. Furthermore, elevated plus maze and open field tests revealed no effect of the intervention on anxiety-related behaviors. Although the current findings do not support a role for the anorectic gut microbiome in cognitive inflexibility, anxiety and reward signaling, future research is required to elucidate if AN-specific microbiota contribute to the pathophysiology of AN.

KEYWORDS: Anorexia Nervosa, gut microbiome, reversal learning, behavioral inhibition, anxiety, reward signaling

Layman's summary

Anorexia Nervosa (AN) is a life-threatening eating disorder, that most commonly develops in adolescence. It is characterized by an intense fear of weight gain, a distorted body image, excessive exercise and self starvation. Unfortunately, the current treatment strategies are not very effective: AN patients often relapse and a substantial part of the patients keeps experiencing symptoms for over 10 years.

For AN patients, it is extremely difficult to change dieting and exercising patterns. This is partially explained by two behavioral traits that individuals with AN are thought to develop: cognitive inflexibility and excessive self-control. Difficulty with being flexible in behavior and choices when circumstances change, is called cognitive inflexibility. This rigidity, combined with excessive self-control, leads AN patients to continue to make unhealthy choices. Researchers have found that AN patients also show cognitive inflexibility and excessive self-control in decisions unrelated to food (for example choices regarding money). It is important to find out why AN patients show aberrant decision making, because this could help improve treatment effectivity.

Because decision making, as well as the motivation to eat food, are regulated by the brain, researchers have attempted to study the brain of AN patients for decades. One signal chemical of particular relevance in these processes is dopamine (DA). DA is released when cells in a brain area called the ventral tegmental area (VTA) are active. DA signaling in the brain seems to be altered during AN development, which may explain changes in decision making and other AN symptoms. What biological mechanisms could cause changes in decision making and DA signaling during the development of AN is so far poorly understood.

Possibly, the bacteria, fungi and viruses (microbiota) inhabiting the gut have a role in the development of AN. These microbiota have an important role in food digestion, but also talk to the brain, affecting mood and behavior. In AN, the gut microbiota composition is perturbed. In this study, we investigated if AN gut microbiota can induce changes in decision making and DA signaling.

To study this, we trained rats to perform neurocognitive tasks for cognitive flexibility and behavioral inhibition and performed surgery on them that allowed for the measurement of the activity of the DA-releasing VTA cells. We then orally administered the rats antibiotics to remove their own gut microbiota and afterwards, transplanted them with fecal samples of three AN patients or healthy controls to give the rats human gut microbiota. We measured cognitive flexibility and behavioral inhibition of the animals before and after treatment using the neurocognitive tasks. At the same time, the activity of VTA cells was measured, using a method called fiber photometry. At the end of the experiment, it was also tested if animals carrying the gut AN microbiota were more anxious than the animals carrying the healthy human gut microbiota by observing their behavior in a maze and open field.

We found no effect of antibiotics treatment and fecal transplant on cognitive flexibility and on the activity of VTA cells. Besides that, AN microbiota-carrying animals were not more anxious than control animals. These findings suggest that healthy rat microbiota and human AN microbiota have no role in cognitive inflexibility, aberrant DA signaling and anxiety. More research is however required to find out if AN microbiota contribute to the development of AN.

Introduction

Anorexia Nervosa (AN) is a life-threatening metabo-psychiatric disorder and is one of the most common chronic disorders in adolescence (van Eeden et al., 2021). It is characterized by an intense fear of weight gain, a distorted body image and compulsive behaviors that facilitate a negative energy balance, such as excessive exercise and extreme caloric restriction. The mortality risk in people followed after treatment for AN is 2-5 times higher than in age- and sex-matched people from the general population (van Hoeken & Hoek, 2020). A combinatorial treatment strategy involving weight rehabilitation and psychotherapy is the current state of the art. Unfortunately, this approach is only moderately effective: relapse rates are extremely high and in around 20% of the patients, symptoms persist for more than 10 years and become chronic (Steinhausen, 2009). While social and psychological causes of AN are better understood and are targeted in treatment, the neurobiological substrates of AN are largely unknown. Neural mechanisms underlying AN persistence and treatment resistance are of particular interest as those may hinder positive long-term therapeutic outcomes. Thus, there is an urgent need for a better understanding of the pathophysiology of AN and novel, more effective treatment approaches.

Two cognitive traits that are consistently reported in anorectic patients and are considered important drivers of treatment resistance are cognitive inflexibility and excessive self-control. Both contribute to aberrant value-based decision making. Cognitive inflexibility entails the inability to change deeply ingrained patterns of thought and behavior (i.e. diet and exercise regimes) in order to adapt to changing conditions (i.e. rapidly declining body weight). In line with clinical observations, a neuropsychological study from Hildebrandt et al. (2015) demonstrated that AN patients show impaired extinguishing and updating of food-neutral stimulus associations in a food-based reversal learning task. Using other neurocognitive paradigms for flexibility, such as the Wisconsin Card Sorting Task and the Brixton Spatial Anticipation Test, it was shown that this cognitive inflexibility in AN is not selective to food-related decisions and persists after weight restoration (Tchanturia et al., 2012; Tchanturia et al., 2011). Similarly, neuropsychological tests for self-control and cognitive inhibition revealed that enhanced self-control in AN patients was not limited to food consumption: AN patients showed more self-control than healthy controls in the Delayed Discounting task with monetary rewards (Steinglass et al., 2012; Decker et al., 2015). Interestingly, aspects of value-based decision making were also shown to be impaired in an animal model of AN, which suggests that physiological changes during AN development contribute to aberrant value-based decision making (Allen et al., 2017). Disentangling what physiology underlies changes in cognitive flexibility and self-control during AN development may help improve treatment effectivity.

Aberrances in value-based decision making, as well as maladaptive food stimulus-reward associations point towards a contribution of the midbrain dopamine (DA) system in AN (Grospe et al., 2018; Verharen et al., 2018; Meye & Adan, 2014). Dopaminergic projections from the ventral tegmental area (VTA) to the ventral striatum – in particular the nucleus accumbens (NAc) – are pivotal for reward processing and reinforcement behaviors including value-based decision making (Boekhoudt et al., 2018). For decades, researchers have attempted to establish a mechanistic role of DA in AN, but no consensus was reached about the direction of the putative change in DA function. Human imaging studies, revealed enhanced activity of the striatum during a prediction error task using fMRI, suggesting that dopaminergic responsivity may be elevated in AN (Frank et al., 2018). Consistent with this hypothesis, another fMRI study showed that women recovered from AN showed enhanced ventral striatal responses to the exposure of both rewarding and aversive food stimuli, compared to age- and BMI-matched controls (Cowdrey et al., 2011). On the other hand, using Positron Emission Tomography (PET) with [^{11}C]raclopride, recovered AN patients were shown to have elevated D2/3 DA

receptor binding in the ventral striatum, which may point towards an increased receptor expression, decreased VTA DA transmission, or a combination of the two (Frank et al., 2005). A study of Broft et al. (2015) did however not replicate these findings in acutely ill AN patients. Studies into the genetic correlates of AN have reported associations between DA-related genes and AN, but none of those have been consistently replicated or were confirmed in large, genome-wide association studies (Duncan et al., 2016). Thus, changes in midbrain DA function have been reported but it remains unclear how the DA system is altered in AN patients.

Animal models are an useful tool to study the mesolimbic pathway in relation to the anorectic phenotype in more detail and under varying conditions. In rodents, chronic food restriction was associated with increased DA reward signaling concomitant with reduced basal DA and enhanced receptor sensitivity (reviewed in Carr, 2020), suggesting starvation may induce low basal striatal DA and DA hypersensitivity in AN. The most popular used animal model for anorexia – the activity-based anorexia model (ABA) – is founded on the finding that pairing limited food access with running wheel availability induces an anorectic phenotype (i.e. weight loss) in susceptible rodents. Increasing striatal DA via selective, chemogenetically induced activation of VTA dopaminergic neurons projecting to the ventral striatum during ABA, was found to rescue the ABA phenotype (Foldi et al., 2017). Furthermore, consistent with the findings of (Frank et al., 2005), mice that showed an anorectic phenotype in the ABA model had upregulated levels of D2 receptor expression in the striatum (Gelegen et al., 2008). Pharmacological blockade of these D2 receptors resulted in reduced vulnerability to ABA (Klenotich et al., 2015), while genetic overexpression of the receptor in the striatum increased ABA vulnerability (Welch et al., 2021). Collectively, these studies suggest DA hypersensitivity, especially of NAc medium spiny neurons expressing the D2 receptor, in the ventral striatum in AN. Hypodopaminergic conditions, usually required to increase DA sensitivity via upregulation of receptor expression and receptor sensitization (reviewed by Kostrzewa et al., 2008), are however not consistently shown in AN models. Possibly, DA function is initially enhanced, reinforcing the development of maladaptive feeding and exercise behaviors, and in a later disease stage is diminished in AN, leading to low basal striatal DA, reward hypersensitivity and cognitive inflexibility (Beeler & Burghardt, 2022). Although it remains to be deciphered what changes in mesolimbic DA action occur during advancing stages of AN, all these works support a central role for midbrain DA dysfunction in AN.

Up until now, it is unknown what biological mechanism causes putative changes in midbrain DA signaling and value-based decision making in AN. One candidate of particular interest is the gut microbiome. The bacteria, viruses, archea and funghi that live in the gut are relatively stable in composition during life (reviewed in Voreades et al., 2014) but can be disrupted by environmental factors, such as starvation in AN. Via communication with the brain (i.e. via systemic hormones, metabolites and immune factors and innervation of the vagal nerve – collectively referred to as the gut microbiota-brain-axis), gut microbiota can affect mood, cognition and behavior (Hata et al., 2019; Heijtz et al., 2011). Mounting evidence suggests that a variety of psychiatric disorders is associated with long-term gut microbiota perturbations. Disorder-specific microbiota may not only be of interest as biomarkers, but also as therapeutic targets for novel non-invasive and inexpensive treatment or prevention approaches. Di Lodovico et al. systematically reviewed and meta-analyzed literature on the gut microbiome in AN and found that the anorectic microbiome has a higher alpha diversity – meaning an increased amount of microbial species and less prominent dominators – and a lower beta diversity – less variability in taxa abundance distributions between samples – compared to the healthy control microbiome (Di Lodovico et al., 2021). The authors reported particularly increased abundances of the genera *Alistipes* and *Parabacteriodes* and a decreased abundance of *Roseburia* in the AN microbiome. If AN-specific dysbiosis is causally linked to the AN phenotype can

be investigated by transplanting animals with fecal samples of anorectic patients and subsequent study of their phenotype. Hata et al. (2019) reconstituted germ-free mice with the microbiota of AN patients and healthy controls. Compared with mice inhabited by a healthy microbiome, the AN microbiome-carrying mice showed a decrease in body weight gain concomitant with reduced food intake. Furthermore, anxiety-related and compulsive behavior, measured by open-field and marble-burying tests, were increased in these mice, suggesting that the microbiome may contribute to anxiety and compulsivity in AN. Although these findings seem promising, the study of AN-specific dysbiosis is still in its infancy and future research is required to conclude if the AN microbiome indeed contributes to AN symptoms.

Whether the AN microbiome contributes to excessive self-control, cognitive inflexibility and aberrant VTA reward signaling, has not yet been studied. There is however some evidence that supports the hypothesis that microbiota can affect these traits. In human subjects with and without obesity, the gut microbiome composition was found to be linked to inhibitory control (Arnoriaga-Rodríguez et al., 2021). The same study also showed that mice transplanted with fecal samples from subjects with obesity and low inhibitory control performed significantly poorer in a reversal learning task than mice reconstituted with healthy control microbiota, suggesting microbiota can also affect cognitive flexibility. Furthermore, emerging evidence suggests a role for the microbiome in the regulation of midbrain DA signaling. Comparisons of germ-free mice with specific-pathogen free mice (carrying a wild type microbiome), revealed that the microbiome affects DA turnover in the striatum and mPFC (Heijtz et al., 2011; Nishino et al., 2013). Additionally, antibiotics-induced intestinal dysbiosis was found to increase DA precursor levels in prefrontal cortex and reduce DA turnover in the amygdala and striatum in Sprague-Lewey rats (Hoban et al., 2016), suggesting rodent dopaminergic circuits are sensitive to changes in gut microbiota. Although these results do not necessarily implicate a role of AN-specific dysbiosis in excessive cognitive control, inflexibility and aberrant DA signaling, they do illustrate that microbiota can exert effects over these AN phenotypical traits.

The current study aims to extinguish this caveat in knowledge by investigating the role of the anorectic microbiome in the development of inflexible value-based decision making, excessive behavioral inhibition and aberrant reward signaling. To this end, recipient tyrosine hydroxylase (TH)-cre rats, surgically injected with cre-dependent GCaMP8s and implanted with optic fibers above the VTA, were pretreated with antibiotics and thereafter reconstituted with the microbiota of three patients with restricting-type AN (AN transplant rats) and three healthy controls (HC transplant rats). Effect of the microbiome on cognitive flexibility and cognitive inhibition were examined using the probabilistic reversal learning task and the Go/NoGo task respectively. Using fiber photometry, activity of the dopaminergic VTA neurons was measured during reinforced and non-reinforced trials of these tasks. By comparing within-subject changes in cognitive flexibility, cognitive inhibition and VTA DA neuronal activity, the effects of antibiotics-induced and AN-specific dysbiosis were studied. Lastly, because AN-specific dysbiosis was associated with increased anxiety-related behaviors and anxiety may affect reinforcement behaviors, anxiogenic or -lytic effects of the intervention were tested employing elevated plus maze and open field tests. AN transplant rats were expected to show impaired performance on the probabilistic reversal learning task, enhanced performance on the Go/NoGo task, increased anxiety-related behaviors and increased reward prediction errors and neural responses to reward.

Materials and methods

Animals

Prior to the experiment, the combination of operant tasks was validated in a pilot experiment using five group-housed, male, Long Evans rats. For the experiment, adult TH-cre Long Evans (♀: n=23, ♂: n=23) rats, bred inhouse, were maintained on a 12-hr light–dark cycle at 22–25 °C with ad libitum access to standard laboratory chow (Standard CRM(e) diet, SDS Diets) and tap water. Prior to surgery, animals were group-housed. After surgery, rats were individually housed in Plexiglas cages (30 x 45 x 35 cm). Males and females were housed in the same room (temperature = 21,5±0,8°C, humidity = 60±2%, light-controlled room with a reversed 12/12 h light-dark schedule (lights on at 7:00pm) and radio, cage enrichment consisted of gnawing stick, tissues and carbon tissue box shelter). Animals were extensively habituated to handling before the start of the experiment. From Exp. Day 15 onwards, animals were mildly food restricted (♀5/♂4 g chow/kg weekly body weight; fixed after start of intervention) to keep them at a stable body weight. When animals substantially lost weight, they were fed extra chow. Animals were fed after operant training or testing, or early in the morning on the other days of the experiment. All experimental manipulations took place during the dark cycle and under red light unless otherwise mentioned. Seven animals were excluded from the study due to untimely death (i.e. post-surgical complications), resulting in a combined total of 39 animals undergoing the experiment. Animals that did not have photometry signal were excluded from the photometry analysis, but not from behavioral analysis (n=3). The experiment was approved by the Committee for Animal Experimentation of the Brain Center Rudolf Magnus, UMC Utrecht, the Netherlands.

Experimental procedures

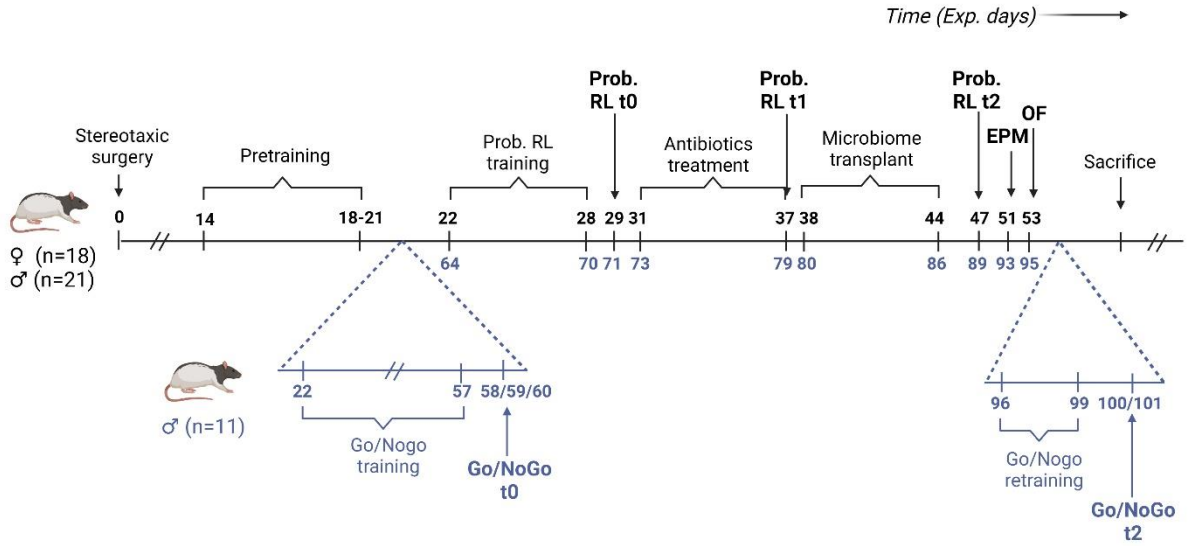

Figure 1. Experimental timeline. *Thirty-nine animals took part in the experiment, of which 11 of the male animals followed an extended experimental protocol that includes the Go/NoGo-task, here shown in blue. Testing days are in bold. Along the x-axis, experimental days are shown – in blue the experimental days of the extended protocol. Abbreviations: Prob. RL = probabilistic reversal learning task, Go/NoGo = Go/NoGo task, EPM = elevated plus maze test, OF = open field test, t0 = baseline measurement, t1 = post-antibiotics measurement, t2 = post-transplant measurement.*

Stereotaxic fiber implantation and virus injection

The rats were anesthetized with intraperitoneal (i.p.) injection of ketamine (75 mg/kg)/dexmedetomidine (0.5 mg/kg). Approximately 30 min before stereotaxic surgery, rats received 5 mg/kg carprofen subcutaneously (s.c.). Anesthetic depth was assessed with a toe pinch. The head was shaved, eye ointment (CAF+) was applied and the rats were placed under a stereotaxic apparatus (UNO BV, The Netherlands) on a heat pat (37°C). By administration of lidocaine (max. 7 mg/kg) locally, the skull was anaesthetized before an incision was made along the midline. Phosphoric acid was applied for 5 min. Craniotomies were then made bilaterally targeting the VTA (A/P -5.60 mm, M/L +1.30 mm, D/V -8.20 mm with a 5 ° angle). To achieve cell-specific GCaMP expression in the TH-cre animals, 1 µl AAV-syn-FLEX-jGCaMP8s-WPRE (Addgene, 1.9 * 10^13 genetic copies/ml, dissolved in PBS) virus was infused at a rate of 0.2 µl per min and allowed to diffuse for 10 min before the needle was withdrawn. Thereafter, the rats were implanted with chronically implantable optic fiber with 400 µm core threaded through plastic ferrules (Thorlabs, CF440-10) above the viral injection site (A/P -5.60 mm, M/L +1.30 mm, D/V -8.10 mm with a 5 ° angle). Fibers were fixated in the skull with 6 screws, metabond glue and dental cement (Fuji PLUS, GC corporation). The incision wound anterior and posterior of the dental cement was closed with sutures. After stereotaxic surgery, the animals were antagonized with atipamezole (2.5 mg/kg) by s.c. injection and received saline for rehydration (1 ml, s.c.). Carprofen was given via drinking water (0.027 mg/mL) for the first 7 days following the surgery. Photometry recordings began a minimum of 3 weeks after surgery to allow sufficient time for stable and robust virus expression.

Intervention

Animals were assigned to one out of four intervention groups: AN transplant, HC transplant, antibiotics and control. Using RandoMice v1.1.6, animals were assigned to a group based on body weight, photometry signal strength (ΔF/F), baseline performance in probabilistic reversal learning (number of relative reversals and self-initiated trials) and if applicable, Go/NoGo performance (%correct Go and NoGo trials), so that the groups did not differ in these parameters before intervention and were of equal size.

Antibiotics treatment

To deplete the host gut microbiome, animals received an antibiotics cocktail consisting of 1.6 mL/kg Metronidazole (40 mg/ml, Flagyl, Sanofi Genzyme), 2 mL/kg Gentamicine (Genta-ject 10%, Dopharma) and 2 mL/kg Vancomycine (Hikma) and 1 mL/kg Ampiciline (20% pro inj., Dopharma) via soft tube oral gavage for 7 consecutive days at ±3.00pm. One group of animals (control, n=10) received a similar volume of water instead of antibiotics.

Microbiome transplant

Microbiome transplant was performed by administration of 0.6 mL human fecal samples dissolved in anaerobic PBS 1x via soft tube oral gavage on the 1st, 2nd, 3rd and 7th day after the end of the antibiotics treatment. A previously performed pilot study had shown that this protocol effectively changes the gut microbiome for at least 10 days. The animals on the extended protocol received an extra booster on the 16th day after the end of the antibiotics treatment. Ten animals received the microbiome of three severe restricting-type AN patients (AN transplant group) and ten animals received that of three healthy controls (HC transplant group). Two groups of animals (control; n=10, and antibiotics group; n = 9) received a similar volume of water instead of fecal sample.

Operant tasks

Operant training started 14 days after surgery. All operant training and testing took place in operant conditioning chambers (Med Associates Inc., USA) equipped with a food receptacle (with infra-red entry detection) flanked by two retractable levers and two cue lights, a house light, habituation cable and an auditory tone generator. White noise (85 dB) was generated in the operant room during training and testing to prevent animals from hearing tones from neighboring chambers. Animals were regularly trained while attached to a habituation cable. Operant trainings and tasks were designed using custom-written MED-PC scripts.

All animals were pretrained to press the lever for a sucrose reward. To familiarize the animals with the conditioning chambers, pretraining started with two 60-min sessions of shaping, during which a 45 mg sucrose pellet (5TUT, TestDiet, USA) was dropped in the food receptacle and one of the cue lights was turned on for 10s every minute. Consumed pellets per session were monitored and animals were left in the chamber for max. 30 min after the end of the training session to eat leftover sucrose pellets. This training phase was completed when animals had consumed > 40 out of 60 sucrose pellets in a session. The animals then proceeded to 60- and 90-min sessions of continuous reinforcement training, during which they only received a sucrose pellet in the food receptacle when they pressed the lever. During these training sessions, each reinforced lever press was followed by a 19.5s inter-trial-interval. If animals did not press the lever, the lever retracted and reappeared every 8 min to guide the animals' attention towards the lever. Continuous reinforcement training was completed when animals had two consecutive sessions with ≥ 50 lever presses, which generally took 2-5 sessions.

Go/NoGo Task

The Go/NoGo paradigm was designed to assess behavioral inhibition and is predominantly used in humans. The task requires individuals to respond to "Go" stimuli and withhold a behavioral response for "NoGo" stimuli. Here, a self-designed equivalent of the human cognitive test suited for rodents is used, that was based on the task of Harrison, Everitt & Robbins (1999). The task itself, as well as the combination with probabilistic reversal learning training, was validated in five pilot animals. The 60-min task consisted of 83 trials, of which 40% Go trials and 60% NoGo trials, presented in a random order. Trial types were signaled to the animal by a 10s auditory tone, which was either high-frequency (5 Hz) or slow-pulse (0.83 Hz)(frequencies adapted from Kolokotroni et al., 2011). Stimulus-response contingencies were counterbalanced. Each trial commenced automatically and started with the illumination of the house light, followed by the discriminative tone for a maximum period of 10s or until a behavioral response was made (i.e. lever press or entry to food receptacle). For Go trials, a lever response during the tone was rewarded with the opportunity to receive a sucrose pellet in the food receptacle. After the lever press, illumination of the cue light above the lever signaled reward availability. If the animal entered the food port within the 5s after cue light illumination, a single sucrose pellet was administered. For NoGo trials, inhibition of the tendency to press the lever for the full 10s duration of the tone was required to obtain a sucrose reward following a food port entry. Incorrect lever responses and premature food port entries (i.e. during the tone) were not reinforced and resulted in a 5s timeout during which the house light was turned off. Pressing the lever at times other than during the presentation of the tone cue had no effect. The number of lever presses during Go and NoGo trials, as well as incorrect head entries in the food receptacle (premature response or omission) and rewards earned, were automatically registered by the software.

Eleven male animals were trained to perform the Go/NoGo task. For the rest of the animals, the Go/NoGo task was removed from the experimental procedure because training was too laborious.

Go/NoGo training consisted of six sessions of Forced Go/NoGo training, ±10 sessions of Iterative-Trial-Go/NoGo training and 19-20 sessions of the final task. Specific for the Forced Go/NoGo sessions, the lever was only presented during the tone of the Go trials, so that incorrect lever responses could not be made. Iterative-Trial-Go/NoGo training differed from the final task only in that Go and NoGo trials were not presented randomly. Instead, incorrect lever presses led to the iteration of the trial type. Completion of Iterative-Trial-Go/NoGo training was achieved when animals had ≥50% NoGo trials in two consecutive sessions.

After training, Go/NoGo baseline measurements were performed (Exp. day 58/59/60). Because animals performed substantially worse during photometry measurement, the averaged data of the last two training sessions per animal was taken as a measure of baseline performance on the Go/Nogo task. Animals with less than 60% Correct Go trials at baseline were excluded from analysis (n=1). Post-transplant Go/NoGo measurements were performed after probabilistic reversal learning training and measurements, intervention, elevated plus maze and open field tests, and 3 sessions of Go/NoGo retraining (Exp. day 100/101).

Probabilistic Reversal Learning Task

The probabilistic reversal learning task is used to assess cognitive flexibility, both in humans and in rodents. In this version of the task, adapted from Verharen, Kentrop, Van der Schuren & Adan (2019), one of the levers is randomly assigned as the high-probability lever and the other as the low-probability lever. Pressing the high-probability lever is reinforced with a sucrose reward in 80% of the cases, and not reinforced in 20% of the cases. Pressing the low-probability lever has a 20% chance of being reinforced and 80% chance of not being reinforced. Selectively when the high-probability lever is pressed eight times consecutively, the levers switch in reinforcement contingencies: from high- to low-probability or the other way around. As this continency switch is not signaled to the animal, it has to infer the reversal from the outcomes of the trials. Adaptive responses (i.e. going to the other lever, that is now the high-probability lever) are considered a sign of cognitive flexibility. During each 90 min-session, animals could take part in as many trials as they wanted (maximum ∼900 trials per session possible). Each trial started with the illumination of the house light and the presentation of the levers. When a lever was pressed, both levers retracted and the house light was turned off. In reinforced trials, a 45 mg sucrose pellet (5TUT, TestDiet, USA) was then dropped into the food receptacle, both cue lights were illuminated and a 0.5s auditory tone was played. As soon as the animal entered the food receptacle (detected by the infra-red movement detector) the cue lights turned off and the next trial started. In non-reinforced trials, a lever press was followed by a 10s timeout, during which there were no cues and the animal had to wait for the next trial in the dark. Behavioral responses and the outcome of the trial (reinforced or not) were automatically registered by the software.

Probabilistic reversal learning training consisted of 7 consecutive training sessions of the task. Performance on the task was measured directly after training (baseline), on the last day of antibiotics treatment (post-antibiotics) and after microbiome transplant with one retraining sessions (post-transplant).

In vivo fiber photometry

The spiking activity of neurons is reflected by their calcium transients. Fiber photometry allows for real-time in vivo measurement of calcium transients in neuronal populations expressing GCaMP (Gunaydin et al., 2014). The hardware set-up used, allowed for simultaneous fiber photometry measurements in two operant chambers with the Doric Neuroscience Studio software (version 5.4.1.1). Under control of a LED driver (950 mA GCaMP and 500 mA isobestic, analog mode, LEDRVP_2CH_1A), two LEDs at 465 nm (GCaMP channel)(LEDC1-B_FC) and 405 nm (control channel for artifactual fluorescence)(LEDC1405_FC) sent light to the optic fiber implants via a Mini Cube (FMC5_AE(405)_AF(420-450)_E1(460-490)_F1(500-550)_S & FMC5_AE(405)_E1(460-490)_F1(500-550)_O(580-650)_S), patch cords (core of 400 µm, FP_400/430/1100-0.57_3m_FCM-MF2.5_LAF) and black-coated ferrules (Precision Fiber Products, SM-CS1140S). LED power was set to 60 uW for the GCaMP channel and to 30 uW for the isobestic (control) channel, measured with an optical power meter (Thorlabs, PM20). Emitted light was collected via the optical fibers and patch cords, passed through the Mini Cube and to a photodetector (AC mode, 2151, Newport), where the two output signals were separated based on modulation frequency (GCaMP: 572.2 Hz, isobestic: 333.8 Hz). Samples were collected at a frequency of 239 samples/s (decimation of 50). MED-PC inputs, required for time-locked activity analysis, and photometry inputs from the photodetectors were integrated by the software via the console.

Recordings were made from VTA TH+ neurons in one hemisphere during the 60-min Go NoGo measurements and 90-min probabilistic reversal learning measurements. Prior to test recordings, signal checks were performed for all animals to conclude which hemisphere was best used for the test recordings (biggest amplitude fluctuations in raw signal F). Fibers were cleaned with 70% ethanol before every recording and baseline F was used to assess if the fiber was adequately connected to the photometry cable. Animals without photometry signal were behaviorally tested in operant chambers with habituation cables.

Data was extracted using custom-written scripts in Python (version 3.7.4). For both the GCaMP and the isobestic channel, autofluorescence was removed from the raw signal (F) by subtraction of the raw signal of the cable. The raw signals were than filtered (butter low pass filter, 6 Hz) and smoothened (50 samples). For the probabilistic reversal learning task, the onset of a lever press and reward were time-locked. For the Go/NoGo task, the onset of the tone was also time-locked. For each time-locked event, the raw signal was converted to ΔF/F for every trial, in which F_0 was the average signal of the -3 to -1s before the event and F_x was the sample value of a given moment between a window of -10 before and 10s after the time-locked event. Reinforced and unreinforced lever presses were analyzed separately.

Elevated Plus Maze

In order to quantify potential anxiolytic or anxiogenic effects of the intervention, elevated plus maze (EPM) tests were carried out on Exp. day 51/93. Because rats are prey animals, they preferentially stay in covered areas. However rats also display curious and exploratory behaviors in new territories. The more time animals spent in the open areas of the EPM, the less anxious they are considered (Walf & Frye, 2007; Pellow et al., 1985).

The EPM tests were conducted in the afternoon, minimally 3h after the rats received their daily chow portion, to prevent food anticipatory activity from affecting the results. The EPM was performed under white light. Five min before testing, animals were brought into the experimental room (with radio and white light) to adapt to the environment. The maze consisted of four 50 cm long arms connected by an open center, arranged in a plus-shape, and was 60 cm elevated above the ground

level. Two arms were open and two arms were enclosed by 40 cm high side walls. The illuminance was 15 lux in the open arms, 5 lux in the closed arms and 10 lux in the center. In the beginning of the test, the rat was positioned in the open center of the maze facing an open arm, after which it could freely move around in the EPM for 10 min. The experiment was recorded with a video camera (SuperLoLux, JVC, TK-C9510E) and analyzed using Noldus Tracking System (version 9). Each rat was tracked at the center of its body. The following parameters were automatically calculated for further analysis: the time spent in the closed and open arms, the number of entries in the closed and open arms and the total distance the animal moved. After every test, the animals were kept in the experimental room for approximately 5 min before getting transferred back to their home cages in the dark. The maze was disinfected with 70% ethanol before every test.

Open field

For robustness of anxiety profiling, open field (OF) tests were carried out two days after the EPM (Exp. Day 53/95). Similar to the EPM, the time spent in the open area of the OF, the center in this case, is considered to be reversely related to anxiety. The arena of the OF was a round field of 80 cm in diameter, enclosed by walls of 30 cm high. The 35% area surrounding the center point of the arena was defined as the center. The illuminance was 55 lux in the borders of the OF and 65 lux in the center of the arena. Similar to the EPM procedure, animals were habituated to the environment for 5 min before being placed in the center the OF and recorded with a video camera (SuperLoLux, JVC, TK-C9510E) for 10 min. Tracking and analysis was performed using Noldus Tracking System (version 9). The following parameters were registered for further analysis: time spent in the center, number of center entries and total distance moved. After every test, rats stayed for 5 min in the experimental room before being returned to their home cages. The arena was disinfected with 70% ethanol before every test.

Stool and vaginal smear sample collection

After every behavioral measurement (probabilistic reversal learning, Go/NoGo, EPM and OF), fresh fecal samples were taken from the rectum of each animal. Materials used for sample collection (i.e. tweezer and petri dish) were cleaned with 70% ethanol in between samples. Stool samples were stored at -80℃ and sent to Max-Planck-Institute for Evolutionary Biology (Plön, Germany) for further analysis. Furthermore, vaginal smears were collected from all female rats on all test days between 4 P.M. and 5 P.M., after behavioral testing, to be able to determine their phase in the estrous cycle according to the protocol of Verharen et al. (2019).

Assessment fiber placement and virus injection site

At the end of the experiment, animals were anaesthetized with an overdose pentobarbital (i.p.) and flushed with PBS before perfusion with 4% paraformaldehyde (PFA) at a rate of approximately 10 mL/min for 5 min. Brains were harvested and kept in 4% PFA overnight, before being transferred to a 30% sucrose solution. After the brains had sunk to the floor of the containers, they were dried and stored in foil at -20℃. Brain tissue sections from bregma -4.56 mm to -6.6 mm were cut at 40 µm and collected in 1x PBS + 0.1% sodiumazide. Free-floating sections were than washed in PBS, blocked for 1hr in a 10% normal goat serum, 0.25% Triton-X in PBS solution, and incubated with 1:1000 chicken anti-GFP (Aves, GFP-1020) and 1:500 rabbit anti-TH (Millipore, AB152) antibodies in 2% NGS in PBS overnight at 4℃. The next day, the sections were washed again in PBS and then incubated with 1:500 goat anti-chicken 488 antibody (Abcam, ab150169) and 1:500 goat anti-rabbit 568 antibody (1:500, Abcam, ab175471) in 2% NGS, 0.25% Triton-X in PBS solution for 1hr at room temperature in the dark. Afterwards, brain sections were washed in PBS, incubated for 20 min in 1 µg/mL DAPI in PBS at room temperature in the dark and then mounted on microscope slides (VWR Avantor, 631-1553), air-dried in the dark and covered with FluorSave (Millipore, 345789) and a coverslip.

Immunohistochemically stained sections were examined under the microscope to determine the fiber placement and virus injection site. Animals with misplaced fibers will be excluded from the photometry analysis.

Statistical analysis

Operant and photometry data were extracted and written to an excel file with custom-written Python scripts (version 3.7.4). Data was sorted in Excel and visualized with GraphPad Prism 9. Statistical analyses were performed in RStudio (version 1.0.153, R version 3.4.1.).

Analysis of probabilistic reversal learning performance was for now limited to the examination of the number of relative reversals (indicating flexibility value-based decision making) and self-initiated trials (indicating motivation) per session. Pre-existing differences between intervention groups for each parameter were examined by the use of Welch two-sample t-tests and one-way ANOVAs. To test for the effect of antibiotics-induced dysbiosis, data of all intervention groups that received antibiotics was pooled (antibiotics group). The antibiotics group was then compared to the control group (no antibiotics) in the change from baseline to post-antibiotics performance employing Mixed Model ANOVAs. Similarly, the effect of AN-specific dysbiosis was tested with Mixed Model ANOVAs, comparing intervention groups in the change from baseline to post-transplant performance. To check if males and females differed in probabilistic reversal learning performance during training and intervention, Mixed Model ANOVAs were employed. For baseline comparisons, two-sample t-tests were used.

Data of the Go/NoGo tasks was preprocessed to obtain the outcome parameters: general performance [(correct Go trials + correct NoGo trials)/total amount of trials*100], %correct Go trials, %correct NoGo trials. Visual inspection of the data led the authors to decide not to statistically test for intervention effects. Instead, training and baseline data from experimental animals was compared to that of pilot animals using a Mixed Model ANOVA and Mann-Whitney U test respectively.

For the analysis of EPM and OF data, one-way ANOVAs were employed to test if treatment groups differed significantly in anxiety-related behaviors. Kruskal-Wallis test was performed if assumptions were not met.

The photometry data was preprocessed by calculation of the averages and the peaks of the ΔF/F signal 0-5s following a reinforced or non-reinforced lever press and 0-2s following the reward. For the non-reinforced lever press only, the peak is defined as the minimum of the ΔF/F signal. Baseline differences in all average and peak parameters were tested using two-sample t-tests for comparison of the antibiotics and no antibiotics group and using one-way ANOVAs for comparison of AN transplant, HC transplant, antibiotics and control animals. Effects of intervention were tested employing Mixed Model ANOVAs.

Assumptions of all two-sample t-tests and One-way ANOVAs were tested using Shapiro-Wilk tests of normality and the Levene's test for homogeneity of variance. Assumptions of Mixed Model ANOVAs were tested using Shapiro's tests (normality), Levene's tests (homogeneity of variances), Box'M statistic (homogeneity of the variance-covariance matrices), Grubb's test (statistical outliers) and Mauchly's test (sphericity). Extreme statistical outliers were removed from the data (photometry analysis antibiotics treatment and fecal transplant: n=2).

Overall, a difference was considered significant when $p<0.05$.

Results

Behavioral results

Microbiome depletion and transplant did not affect flexible value-based decision making

All animals learned to flexibly respond to reversals of the reinforcement contingencies during training of the probabilistic reversal learning task (Fig. 2C). Antibiotics and no antibiotics groups did not differ in the number of self-initiated trials ($t(14.934) = -1.262$, $p>0.05$)(Fig. 2D) and relative reversals ($t(14.557) = -0.5188$, $p>0.05$)(Fig 2E) at baseline. Microbiome depletion with antibiotics had no effect on the number of self-initiated trials (*intervention*: $F(1,37) = 2.526$, $p>0.05$, *time*: $F(1,37) = 0.245$, $p>0.05$, *intervention x time*: $F(1,37) = 0.003$, $p>0.05$)(Fig. 2H) and neither on the number of relative reversals (*intervention*: $F(1,37) = 1.221$, $p>0.05$, *time*: $F(1,37) = 1.878$, $p>0.05$, *intervention x time*: $F(1,37) = 0.172$, $p>0.05$)(Fig. 2I).

AN transplant, HC transplant, antibiotics and control animals did not differ in the number of self-initiated trials ($F(3,34) = 0.632$, $p>0.05$)(Fig. 2F) and relative reversals at baseline ($F(3,34) = 0.556$, $p>0.05$)(Fig.2G). Transplantation with human fecal samples also had no effect on the number of self-initiated trials (*intervention*: $F(3,34) = 0.619$, $p>0.05$, *intervention x time*: $F(3,34) = 0.499$, $p>0.05$)(Fig. 2J). However, there was a significant effect of *time* on number of trials when comparing baseline data to post-transplant data ($F(1,34) = 24.600$, $p<0.001$). Microbiome transplantation had no effect on relative reversals (*intervention*: $F(3,34) = 0.392$, $p>0.05$, *time*: $F(1,34) = 2.569$, $p>0.05$, *intervention x time*: $F(3,34) = 0.525$, $p>0.05$)(Fig. 2K).

For descriptive statistics, see supplemental Table 1.

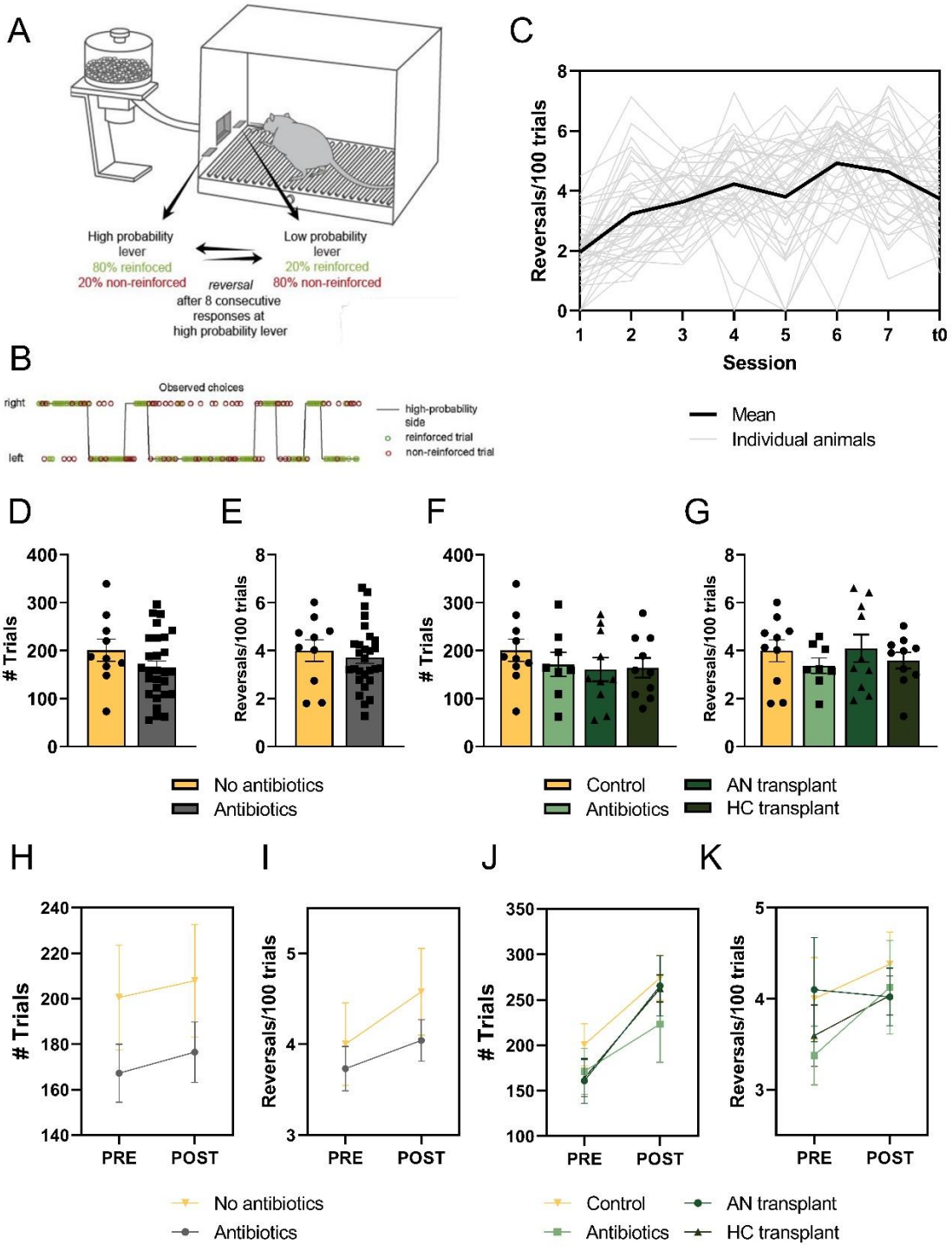

Figure 2. Microbiome depletion and transplant did not affect flexible value-based decision making. *(A) Schematic representation of the Probabilistic Reversal Learning task, adapted from Verharen et al. (2019). (B) Example trial-to-trial data of flexible value-based decision making behavior during the task, adapted from Verharen et al. (2019). (C) Acquisition of probabilistic reversal learning performance during training sessions (1-7) and baseline measurement (t0). (D) Number of self-initiated trials and (E) relative reversals at baseline of antibiotics and no antibiotics (control) animals. (F) Number of self-initiated trials and (G) relative reversals at baseline of all intervention groups. (H) Change in the number of self-initiated trials and (I) relative reversals from baseline to post-antibiotics measurement in antibiotics- and control-treated animals. (J) Change in the number of self-initiated trials and relative reversals (K) from baseline to post-transplant measurement in AN transplant, HC transplant, antibiotics and control animals. For figures 2D-K, values represent mean ± SEM. No significant group differences were reported in any of the plotted parameters. Abbreviations: AN = anorexia nervosa, HC = healthy control, t0 = baseline measurement.*

Effects microbiome depletion and transplant on flexible value-based decision are independent of sex

Based on earlier research, sex and estrous cycle hormones in female rats were expected to affect the number of self-initiated trials in the probabilistic reversal learning task (Verharen et al., 2019). Therefore, additional analyses on training and baseline data were performed to test for an effect of sex on probabilistic reversal learning performance. Mixed Model ANOVA revealed no effect of *sex* on relative reversals during the training phase (*sex*: F(1,37) = 1.157, p>0.05, *time*: F(7,259) = 21.097, p<0.001, *sex x time*: F(7,259) = 1.830, p>0.05) (Fig. 3A). Additionally, males and females did not differ in the number of self-initiated trials (Kruskal-Wallis $\chi^2(1)$= 0.302, p>0.05) and relative reversals (F(1,37) = 1.325, p>0.05) at baseline.

Since females may additionally be more susceptible to the development of anorectic phenotypes (van Eeden et al., 2021), previously reported Mixed Model ANOVAs were rerun with *sex* as extra between-subject to test for an interaction between *sex* and *intervention.* This did not yield significant interaction effects of *sex* and *antibiotics treatment* on self-initiated trials (*sex*: F(1,35) = 0.538, p>0.05, *sex x time*: F(1,35) = 0.037, p>0.05, *sex x intervention*: F(1,35) = 0.002, p>0.05, *sex x intervention x time*: F(1,35) = 0.037, p>0.05). There was no interaction effect of antibiotics treatment and sex on relative reversals over time (*sex*: F(1,35) = 3.406, p>0.05, *sex x time*: F(1,35) = 1.773, p>0.05, *sex x intervention x time*: F(1,35) = 1.481, p>0.05). However, there was an interaction between *sex* and *antibiotics intervention* on relative reversals, irrespective of time (*sex x intervention*: F(1,35) = 5.335, p<0.05).

Furthermore, no interaction between fecal transplant and *sex* was found on the number of self-initiated trials (*sex*: F(1,30) = 1.403, p>0.05, *sex x time*: F(1,30) = 0.299, p>0.05, *sex x intervention*: F(3,30) = 0.728, p>0.05, *sex x intervention x time*: F(3,30) = 0.773, p>0.05) and relative reversals (*sex*: F(1,30) = 1.415, p>0.05, *sex x time*: F(1,30) = 0.566, p>0.05, *sex x intervention*: F(3,30) = 1.937, p>0.05, *sex x intervention x time*: F(3,30) = 1.498, p>0.05).

Adding an extra between-subject factor in the Mixed Model ANOVAs did subtly change statistical outcomes on the *intervention*, *time* and *intervention x time* effects, but that did not affect statistical significance.

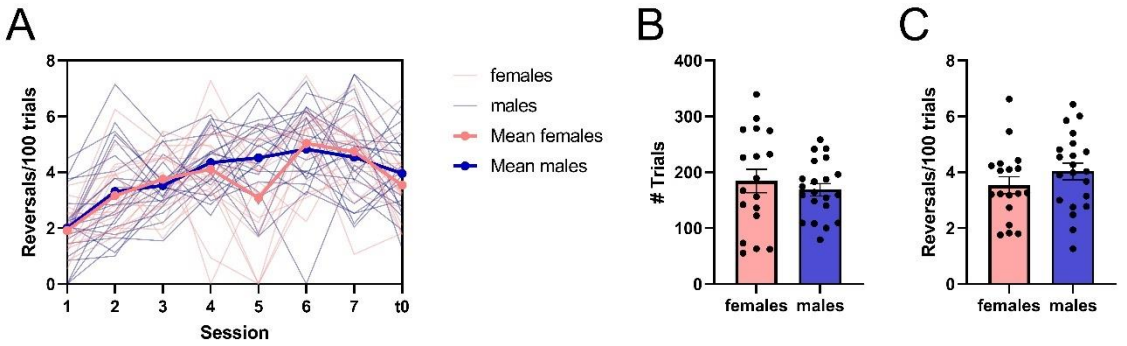

Figure 3. No sex differences in the acquisition and baseline performance on the probabilistic reversal learning task for flexible value-based decision making. *(A) Relative reversals per training session (1-7) and during baseline measurement (t0). (B) Number of self-initiated trials and (C) relative reversals at baseline of male and female rats. For figures 3B-C, bars represent mean ± SEM, dots represent individual data points. No significant group differences were reported in any of the plotted parameters.*

Effect of anorectic microbiome transplant on behavioral inhibition remains to be elucidated

Despite efforts to validate the Go/NoGo task for behavioral inhibition in a pilot group, data from the experimental animals on the Go/NoGo task is inappropriate for adequate interpretation of intervention effects on behavioral inhibition due to the small sample sizes ($n \leq 3$ per group), big variance in performance and poor baseline performance in most animals. To illustrate performance accuracy of the experimental animals, data from their training sessions was compared to that of the pilot group (Fig. 4). Similar to the pilot animals, experimental animals stabilized in performance on NoGo trials after approximately 12-13 training sessions (Fig. 4A). Performance on the NoGo trials was lower in experimental animals than in the pilot animals on training sessions 16-18, although statistical significant was not reached ($W = 15$, $p>0.05$)(Fig. 4B). Statistical insignificance is presumably due to big variance in the data of both groups.

Given the large between- and within-subject variance in the Go/NoGo data, the authors conclude that the task was too difficult to learn for the animals, and is therefore an inadequate measure of behavioral inhibition. Thus, observations regarding Go/NoGo performance over the course of the microbiome intervention are described below, but are not considered informative about microbiota-induced effects on behavioral inhibition.

Overall performance on the Go/NoGo task was around 57% correct trials on average for all intervention groups at baseline, and was not substantially altered in animals reconstituted with a human microbiome, but was somewhat decreased in antibiotics and control animals after the intervention (Fig. 5A). The %correct NoGo trials, putatively reflective of behavioral inhibition capacity, was increased in the HC transplant group, and decreased in the antibiotics and control group by the intervention (Fig. 5B). For the HC transplant, antibiotics and control groups, this change in %correct NoGo trials can be attributed to a change in premature lever presses (Fig. 5C). These intervention groups did not differ in premature head entries before and after intervention (Fig. 5D). The AN transplant groups seems to have increased premature lever presses, but substantially decreased premature head entries (irrespective of trial type), which together resulted in a similar %correct NoGo trials after intervention.

For descriptive statistics, see supplemental Table 2.

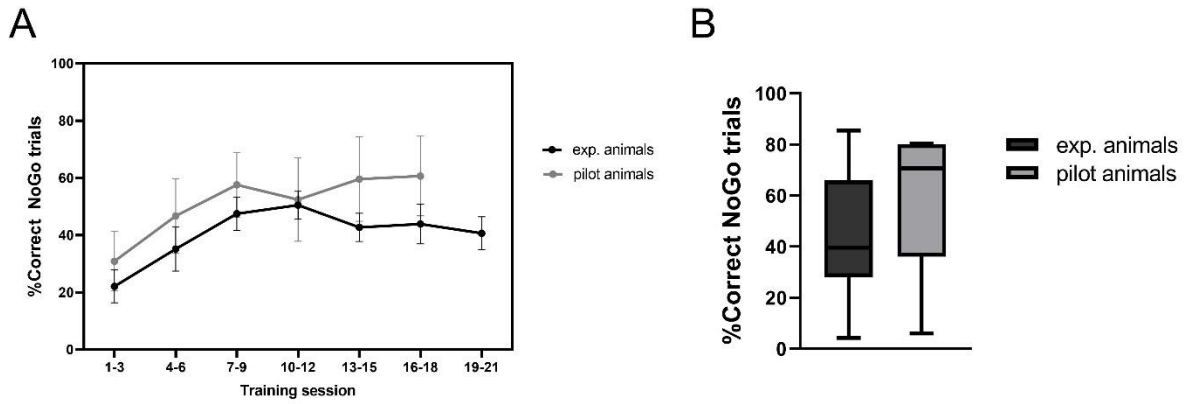

Figure 4. Go/NoGo performance acquisition compared to pilot animals. *(A) Performance on the NoGo trials over the training sessions (averaged per three sessions) of experimental animals (n=11) and pilot animals (n=5). Values represent mean ± SEM. (B) Performance on NoGo trials during training session 16-18 (averaged) of experimental animals and pilot animals. Box plots indicate median ± IQR. Error bars indicate minimum and maximum. Abbreviations: exp. = experimental. No significant group differences were reported in any of the plotted parameters.*

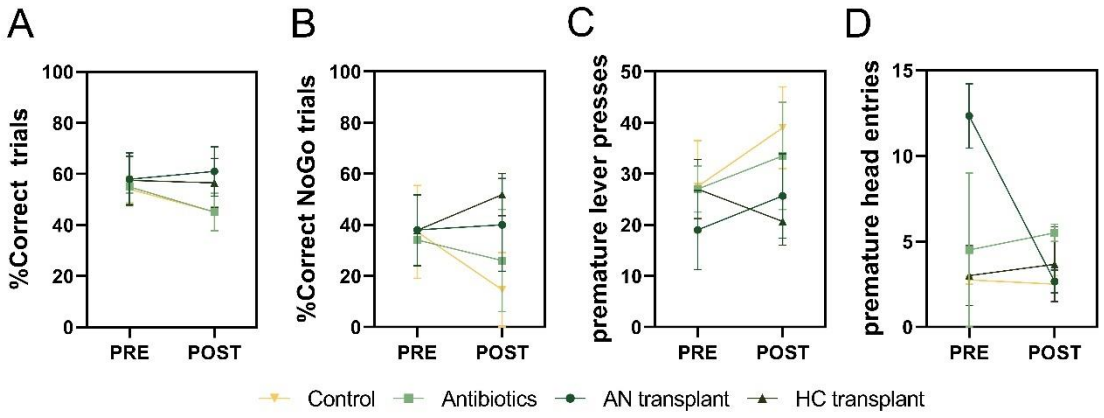

Figure 5. Fecal transplant of anorectic gut microbiome may affect Go/NoGo performance, but further research is required to elucidate to role of the anorectic gut microbiome in behavioral inhibition. *(A) Change from baseline to post-transplant measurement in overall performance, irrespective of trial type (Go or NoGo). (B) Change from baseline to post-transplant measurement in performance on the NoGo trials, during which the animals had to withhold their lever response to earn a reward. (C) Change from baseline to post-transplant measurement in the number of lever presses during NoGo trials. (D) Change from baseline to post-transplant measurement in the number of premature head entries during Go and NoGo trials. For figures 4A-D, values represent mean ± SEM. Group differences were not statistically tested. Abbreviations: AN = anorexia nervosa, HC = healthy control.*

Microbiome transplant did not affect anxiety-related behavior in the elevated plus maze and open field

Despite extensive habituation to handling, novel situations and environments, all animals displayed anxious behavior (i.e. freezing, jumpy behavior, increased defecation) in both the EPM and the OF.

Post-transplant measurements of anxiety-related behavior in the EPM yielded no differences in time spent in the open arms ($F(3,34) = 0.331$, $p>0.05$)(Fig. 6B), open arm entries ($F(3,34) = 0.62$, $p>0.05$)(Fig. 6C) and total distance moved in the EPM ($F(3,34) = 0.408$, $p>0.05$)(Fig. 6D). Similarly, no differences were found between the intervention groups in time spent in the center of the OF ($F(3,34) = 0.796$, $p>0.05$)(Fig. 6F), OF center entries ($F(3,34) = 0.469$, $p>0.05$)(Fig. 6G), latency to first center visit (Kruskal-Wallis $\chi^2(3)= 0.754$, $p>0.05$) and distance moved in the OF ($F(3,34) = 0.591$, $p>0.05$)(Fig. 6H). Thus, no differences between the intervention groups were observed in any of the EPM and OF parameters.

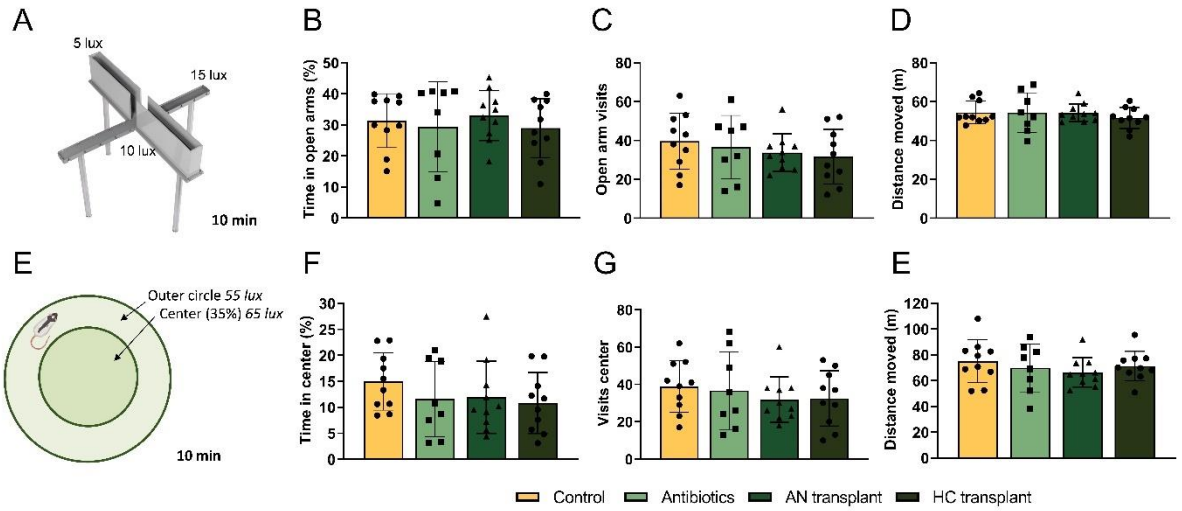

Figure 6. Transplant of anorectic gut microbiome did not affect anxiety-related behavior in the elevated plus maze (EPM) and open field (OF). *(A) Schematic representation of the EPM with illuminance per compartment of the arena and total duration of the EPM exposure. (B) Time spent in the open arms of the EPM per intervention group. (C) Number of visits of the open arms of the EPM per intervention group. (D) The total distance moved in the EPM arena per intervention group. (E) Schematic representation of the OF with illuminance per compartment of the arena and total duration of the OF exposure. (F) Time spent in the center of the OF per intervention group. (G) Number of visits to the center of the OF per intervention group. (H) The total distance moved in the entire OF arena per intervention group. Values represent mean±SEM. No significant group differences were reported in any of the plotted parameters. Abbreviations: AN = anorexia nervosa, HC = healthy control.*

Photometry results

During the probabilistic reversal learning task, neuronal responses to lever presses and sucrose pellet drops were recorded for both reinforced and non-reinforced trials, measuring from the TH-expressing neurons in the VTA (Fig. 7A). Recordings were also made during the Go/NoGo task, but this data is not yet analyzed. Based on the immunohistochemically stained slices (example in Fig. 7B), fiber ends and virus expression will be localized for each animal to verify recordings were selectively made from VTA DA neurons. This verification process is still ongoing, but none of the animals seem to have had fibers ending in the substantia nigra, so exclusions based on fiber misplacement are not expected. In correspondence with literature on VTA DA neural responsivity (Eshel, Tian, Bukwich & Uchida, 2016), ΔF/F signal was higher in reinforced lever presses compared to non-reinforced lever presses in all animals (Fig. 7C-D).

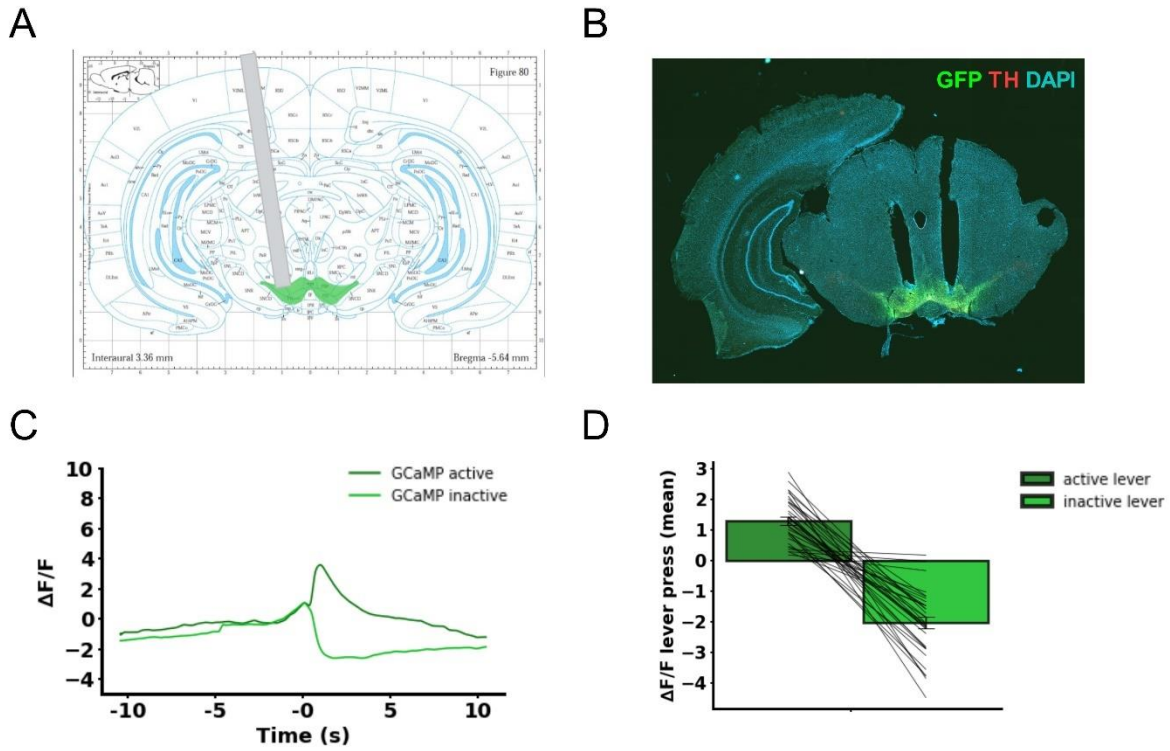

Figure 7. Validation of photometry data. *(A) Schematic representation of intended fiber placement. Animals were implanted with fiber ends bilaterally, but were measured from the most optimal hemisphere only. (B) Example of immunohistochemically stained slice, used to localize the relevant fiber end and virus expression. In this microscopic picture, both fibers would be considered accurately aimed at the VTA. (C) Neural response of measured VTA dopaminergic neurons to reinforced and nonreinforced lever presses before intervention, average of all animals included in photometry analysis. (D) Baseline measurement of mean ΔF/F signal following reinforced lever presses compared to non-reinforced lever presses. Bars and error bars represent mean±SEM, lines represent averages of individual animals. In Fig. 7C-D, active refers to reinforced lever presses and inactive to non-reinforced lever presses.*

Depletion of the gut microbiome with antibiotics did not affect neural responses of VTA dopaminergic neurons to reward prediction errors and rewards

Antibiotics and no antibiotics animals did not differ in the average ΔF/F signal following a reinforced lever press ($t(32) = 1.469$, $p>0.05$), non-reinforced lever press ($t(32) = -0.197$, $p>0.05$) and reward ($W = 162$, $p>0.05$), and neither in peak ΔF/F signal following a reinforced lever press ($t(32) = 0.822$, $p>0.05$), non-reinforced lever press ($t(32) = -0.352$, $p>0.05$) and reward ($t(32) = -0.234$, $p>0.05$), prior to antibiotics treatment.

Microbiome depletion with antibiotics had no effect on the average ΔF/F signal following a reinforced lever press (*intervention*: $F(1,32) = 1.775$, $p>0.05$, *time*: $F(1,32) = 0.149$, $p>0.05$, *intervention x time*: $F(1,32) = 0.030$, $p>0.05$)(Fig. 8B) and on the peak ΔF/F signal following a reinforced lever press (*intervention*: $F(1,32) = 1.166$, $p>0.05$, *time*: $F(1,32) = 0.069$, $p>0.05$, *intervention x time*: $F(1,32) = 0.531$, $p>0.05$)(Fig. 8C). Similarly, no effect of antibiotics treatment was found on the average ΔF/F signal following a non-reinforced lever press (*intervention*: $F(1,32) = 0.023$, $p>0.05$, *time*: $F(1,32) = 0.002$, $p>0.05$, *intervention x time*: $F(1,32) = 0.018$, $p>0.05$)(Fig. 8E) and on the peak ΔF/F signal following a non-reinforced lever press (*intervention*: $F(1,32) = 0.096$, $p>0.05$, *time*: $F(1,32) = 0.014$, $p>0.05$, *intervention x time*: $F(1,32) = 0.048$, $p>0.05$)(Fig. 8F). Lastly, antibiotics-induced dysbiosis had no effect on the average ΔF/F signal following a reward (*intervention*: $F(1,32) = 0.918$, $p>0.05$, *time*: $F(1,32) = 0.350$, $p>0.05$, *intervention x time*: $F(1,32) = 0.078$, $p>0.05$)(Fig. 8H) and on the peak ΔF/F signal following a reward (*intervention*: $F(1,32) = 0.105$, $p>0.05$, *time*: $F(1,32) = 0.167$, $p>0.05$, *intervention x time*: $F(1,32) = 0.059$, $p>0.05$)(Fig. 8I).

Reconstitution with AN microbiota did not affect neural responses of VTA dopaminergic neurons to sucrose reward prediction and receival

Prior to the intervention, AN transplant, HC transplant, antibiotics and control animals did not differ in the average ΔF/F signal following a reinforced lever press ($F(3,29) = 0.858$, $p>0.05$), non-reinforced lever press ($F(3,29) = 0.311$, $p<0.05$) and reward ($F(3,29) = 1.157$, $p>0.05$), and neither in peak ΔF/F signal following a reinforced lever press ($F(3,29) = 0.344$, $p>0.05$), non-reinforced lever press ($F(3,29) = 0.288$, $p>0.05$) and reward ($F(3,29) = 0.270$, $p>0.05$).

Microbiome transplantation had no effect on the average ΔF/F signal following a reinforced lever press (*intervention*: $F(3,29) = 1.528$, $p>0.05$, *time*: $F(1,29) = 1.240$, $p>0.05$, *intervention x time*: $F(3,29) = 0.629$, $p>0.05$)(Fig. 9B) and on the peak ΔF/F signal following a reinforced lever press (*intervention*: $F(3,29) = 0.898$, $p>0.05$, *time*: $F(1,29) = 0.001$, $p>0.05$, *intervention x time*: $F(3,29) = 0.846$, $p>0.05$)(Fig. 9C). Furthermore, microbiome transplantation had no effect on the average ΔF/F signal following a non-reinforced lever press (*intervention*: $F(3,29) = 0.303$, $p>0.05$, *intervention x time*: $F(3,29) = 1.552$, $p>0.05$)(Fig. 9E). However, irrespective of *intervention*, there was a significant effect of *time* on the average ΔF/F signal following a non-reinforced lever press ($F(1,29) = 5.597$, $p<0.05$). This *time* effect could not be attributed to a *time* effect on the peak of the ΔF/F signal ($F(1,29) = 3.977$, $p>0.05$). The peak ΔF/F signal following a non-reinforced lever press was not altered by microbiome transplantation (*intervention*: $F(3,29) = 0.376$, $p>0.05$, *intervention x time*: $F(3,29) = 0.906$, $p>0.05$)(Fig. 9F). Lastly, no effect of the fecal transplant was observed on the average ΔF/F signal following a reward (*intervention*: $F(3,29) = 1.546$, $p>0.05$, *time*: $F(1,29) = 3.645$, $p>0.05$, *intervention x time*: $F(3,29) = 0.799$, $p>0.05$)(Fig. 9H) and on the peak ΔF/F signal following a reward (*intervention*: $F(3,29) = 0.392$, $p>0.05$, *time*: $F(1,29) = 2.748$, $p>0.05$, *intervention x time*: $F(3,29) = 0.801$, $p>0.05$)(Fig. 9I).

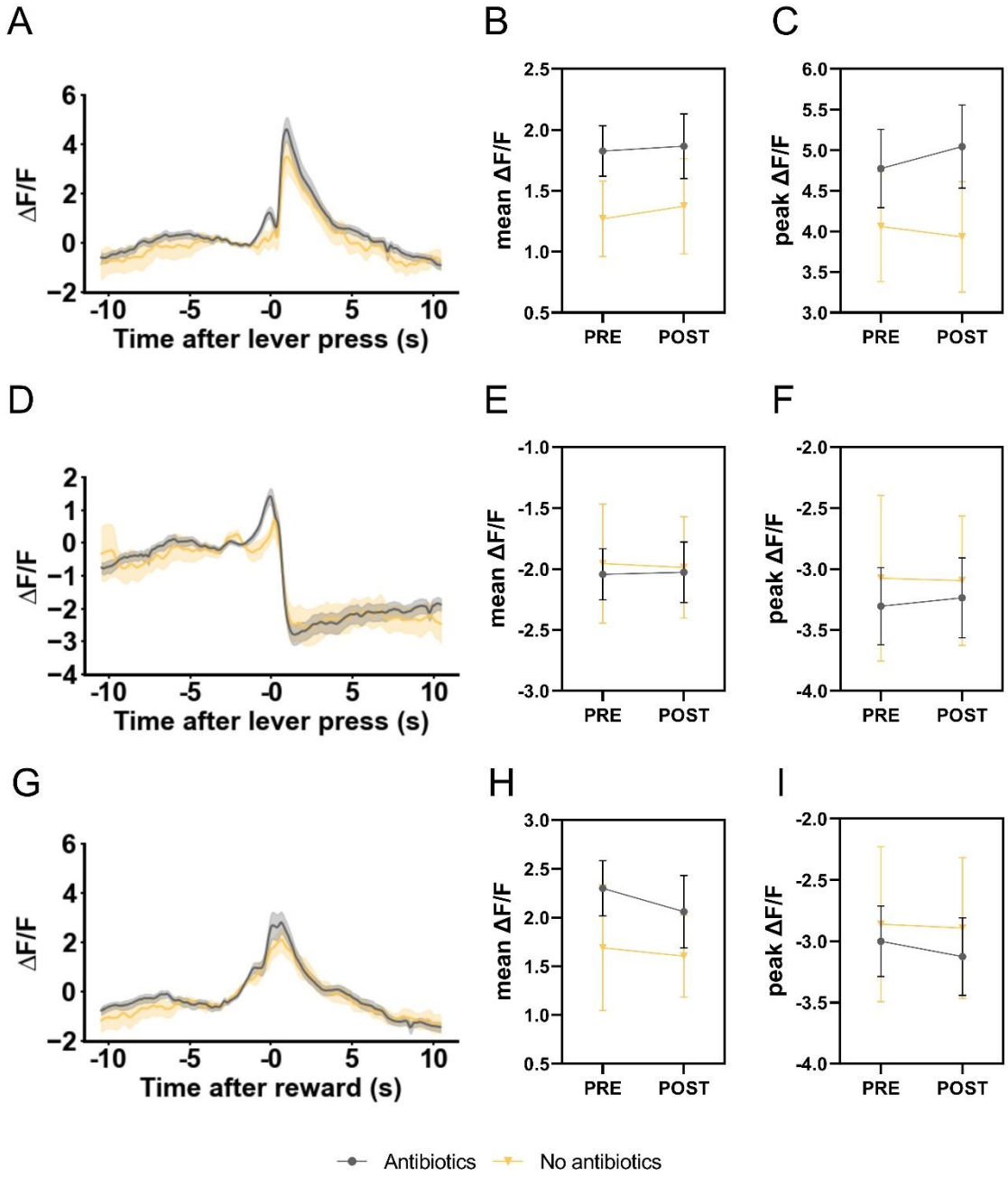

Figure 8. Microbiome depletion with antibiotics did not affect neural responses of VTA dopaminergic neurons to reward and reward prediction. *(A) Phasic firing fluctuations of VTA dopaminergic neurons of antibiotics-treated and control animals, time-locked to a reinforced lever press. (B) Change from baseline to post-antibiotics measurement in average and (C) peak ΔF/F signal in the five seconds following a reinforced lever press in antibiotics-treated and control animals. (D) Phasic firing fluctuations of VTA dopaminergic neurons antibiotics-treated and control animals, time-locked to a non-reinforced lever press, averaged per group. (E) Change from baseline to post-antibiotics measurement in average and (F) peak ΔF/F signal in the five seconds following a non-reinforced lever press in antibiotics-treated and control animals. (G) Phasic firing fluctuations of VTA dopaminergic neurons of antibiotics-treated and control animals, time-locked to reward. (H) Change from baseline to post-antibiotics measurement in average and (I) peak ΔF/F signal in the two seconds following a reward in antibiotics-treated and control animals. Values represent mean±SEM. No significant group differences were reported in any of the plotted parameters.*

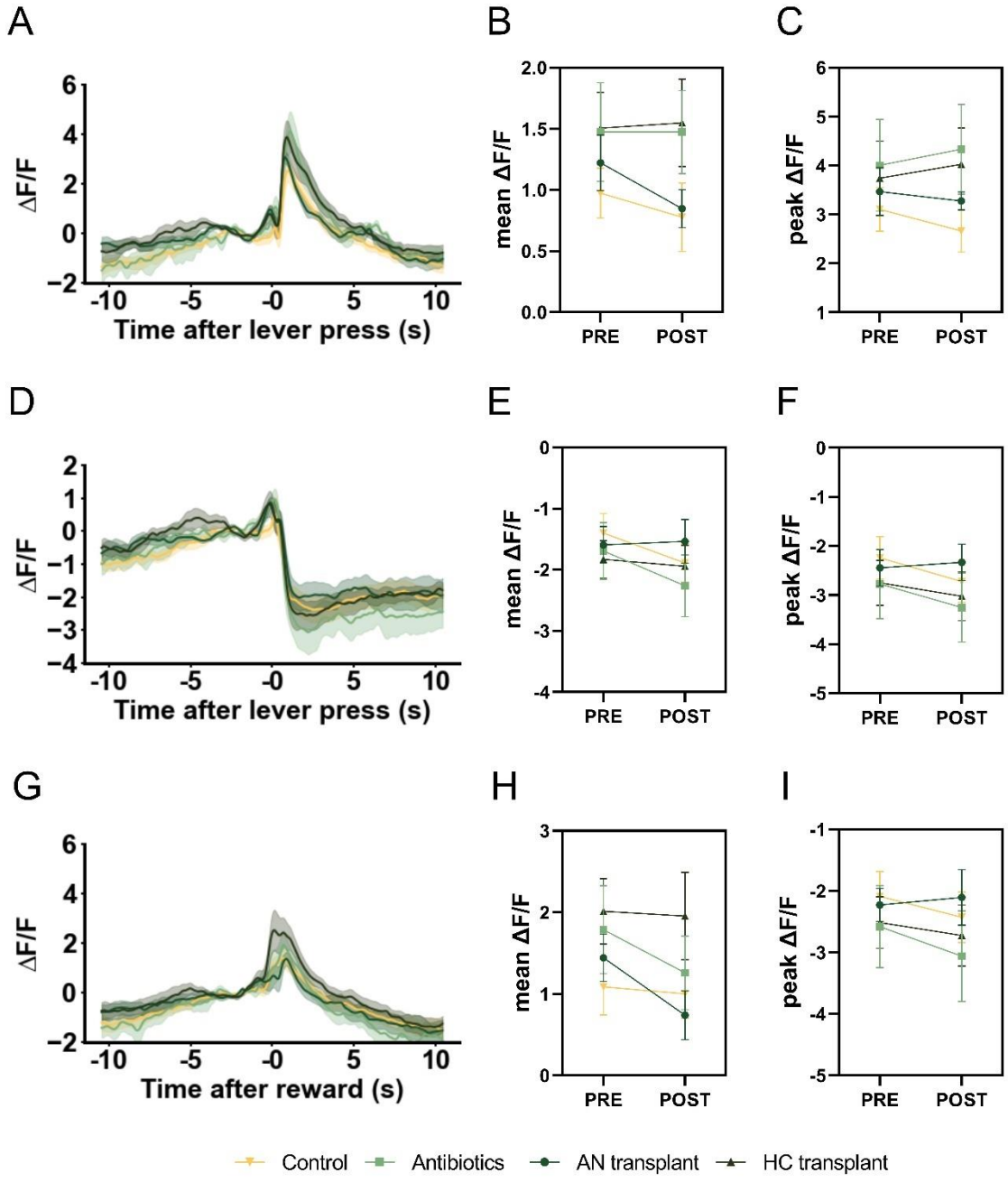

Figure 9. Microbiome transplant did not affect neural responses of VTA dopaminergic neurons to reward and reward prediction. *(A) Phasic firing fluctuations of VTA dopaminergic neurons per intervention group, time-locked to a reinforced lever press. (B) Change from baseline to post-transplant measurement in average and (C) peak ΔF/F signal in the five seconds following a reinforced lever press. (D) Phasic firing fluctuations of VTA dopaminergic neurons per intervention group, time-locked to a non-reinforced lever press. (E) Change from baseline to post-transplant measurement in average and (F) peak ΔF/F signal in the five seconds following a non-reinforced lever press. (G) Phasic firing fluctuations of VTA dopaminergic neurons per intervention group, time-locked to reward. (H) Change from baseline to post-transplant measurement in average and (I) peak ΔF/F signal in the two seconds following a reward. Values represent mean±SEM. No significant group differences were reported in any of the plotted parameters. Abbreviations: AN = anorexia nervosa, HC = healthy control.*

Discussion

Here, we show that both depletion of the host microbiome with antibiotics and reconstitution with human anorectic and healthy control microbiomes have no effect on the number of self-initiated trials and relative reversals of recipient rats in the probabilistic reversal learning task. Sex of the recipient rats did not affect these intervention results. Furthermore, no differences between AN transplant, HC transplant, antibiotics and control groups were reported in anxiety-related behaviors in the elevated plus maze and open field. Lastly, we show no effect of antibiotics treatment or fecal transplant on VTA DA neuron responses to reinforced and non-reinforced lever presses as well as sucrose reward receival. These findings indicate that antibiotics-induced and AN-specific dysbiosis are insufficient to induce changes in reversal learning performance, anxiety-related behavior and VTA DA neural responses to reward, reward anticipation and reward prediction error in a sucrose operant task. Thus, there is no report of any evidence suggesting a role of the anorectic gut microbiome in cognitive inflexibility, anxiety and reward signaling.

This study is one of the first to investigate a causal relationship between AN-specific dysbiosis and AN phenotype development. When Hata and colleagues (2019) revealed effects of the AN microbiome on body weight gain, food intake and anxiety-related and compulsive behavior, the role of AN-specific dysbiosis in AN pathophysiology seemed promising. However, a study of Glenny et al. (2021) into AN-microbiota-induced changes in body weight gain and food intake did not replicate their finding. The current study reports no behavioral effects of AN-specific dysbiosis in rats with humanized gut microbiomes. Discrepancies between the study outcomes may be due to the use of different recipient animals (i.e. strain and developmental stage differences), different donors and different models for humanization of the animals gut microbiome. The complexity of the microbiome and its interaction with the brain complicates the study of potential microbiota-associated behavioral effects. AN-specific dysbiosis may require interaction with other systems or metabolites involved in AN pathophysiology to affect AN phenotype development (e.g., HPA-axis metabolites after exposure to acute or chronic stress). Furthermore, AN-specific dysbiosis may only play a role in the persistence or exaggeration of AN phenotypes and not in the development thereof, as some hypothesize these are two distinct neurobiological stages in AN (Beeler & Burghardt, 2022). Clearly, the field is still in its infancy and more research is required to elucidate if AN-specific dysbiosis has a role in the pathophysiology of the disorder.

Our findings indicate that AN-specific dysbiosis does not affect cognitive inflexibility, anxiety and VTA reward signaling. However, the following methodological limitations should be taken into consideration.

Firstly, it has not yet been verified that the gut microbiomes of the animals were altered by our intervention and to what extent. Previously performed pilot studies testing this intervention protocol in adolescent female Wistar rats, revealed that antibiotics treatment induces substantial decreases in alpha diversity (number and distribution of bacterial strains) and that 20% of the microbiome was humanized after fecal transplant. Thus, a considerable amount of microbiota from the donor samples does not survive the experimental procedure, and this may include potential AN-specific bacterial strains that are essential in microbiota-induced AN phenotype development. Furthermore, the intervention method has not yet been piloted in adult male and female Long Evans rats. Possibly, the intervention is less effective than in the pilot. In particular the adult males, whose body weight was substantially larger than of the pilot animals, may require bigger doses of antibiotics and fecal transplant for the same effectiveness. Based on the pilot results, substantial changes in the gut microbiomes of the animals are expected after each intervention phase, but remain to be quantified.

Secondly, the power to detect small-sized effects of the anorectic microbiome on behavior and reward signaling was limited. The sample sizes were small and fecal samples of only three patients with restricting-type AN and three healthy controls were used. Given the diversity in microbiome composition across the AN patient population and especially across a healthy control population, results may not be generalizable. More importantly, phenotypic profiles (i.e. cognitive flexibility and anxiety personality traits) of the donors lack. The latter is of particular relevance because both the AN patient population and the healthy control population are likely heterogenous in the expression of the studied behavioral phenotypes.

Thirdly, potential effects of AN-specific dysbiosis may be more pronounced in female adolescents, whose brain regions (e.g., prefrontal cortex) involved in behavioral inhibition, reward processing and value-based decision making are still developing (reviewed in Hartley & Somerville, 2015). AN typically develops during adolescence and young adulthood, is more prevalent in women (van Eeden et al., 2021). From experience in the lab, it is also known that adolescent female rodents are most susceptible to ABA. It may be of interest to replicate the experiment in adolescent female rats. Along the same line of reasoning, it may be of interest to select young females that show poor baseline probabilistic reversal learning performance, high levels of wheel running activity, high levels of anxiety-related behaviors in the EPM or weight-recovered females that were found to be most susceptible to ABA (Pjetri et al., 2012).

Lastly, it can be argued that AN-specific dysbiosis may have induced AN phenotypes in the current experiment, without affecting the measured outcome parameters. Although cognitive inflexibility, enhanced behavioral control, anxiety-related symptoms and aberrant reward experiences of food stimuli are consistently reported in AN patients, these AN features may not be captured by the probabilistic reversal learning task, Go/NoGo task, EPM, OF and photometry approaches in this experiment.

Probabilistic reversal learning task for cognitive inflexibility. Attentional set shifting and reversal learning are regularly used intermingled when describing cognitive inflexibility in AN. However, these domains of cognitive flexibility are very distinct in that reversal learning, but not set-shifting, measures the ability to update responses after reinforcement feedback. Looking at reversal learning paradigms only, findings of impaired performance in AN patients are inconsistent. While Hildebrandt et al. (2015) showed impaired reversal learning of acutely ill AN patients, others were unable to replicate these findings (Adoue et al., 2015) (Geisler et al., 2017). Hildebrandt and colleagues were the only one to use a food-based reversal learning paradigm, which may suggest that reversal learning is only impaired in AN in a food-specific context. In contrast to the other studies, the reinforcement stimuli (food pictures) used in the study of Hildebrandt are negative reinforcers for the acutely ill AN patients and it is hypothesized that AN patients are particularly sensitive to negative reinforcement and punishment (Geisler et al., 2017; Bernardoni et al., 2018). Moreover, the AN patients had enhanced emotional salience for the reinforcer used in the study of Hildebrandt et al., which likely affects reinforcement behaviors such as reversal learning. Thus, impaired reversal learning may depend on the increased salience and negative experience of the reinforcer, while these criteria may not have been met in the current experiment. Furthermore, weight status and metabolic state may be crucial mediators of AN associations with impaired reversal learning, and could not have differed between intervention groups in this body weight-controlled experimental design.

Go/NoGo task for excessive behavioral inhibition. It was impossible to assess the effect of host microbiome depletion and AN-specific dysbiosis on cognitive inhibition using the Go/NoGo task due

to the inability to obtain stable baseline performance in the animals. The use of a less complex task, such as the five-choice serial reaction time task, is recommended for further research into rodent behavioral inhibitory capacities.

Elevated plus maze and open field tests for increased anxiety. EPM and OF field tests are well-established paradigms for general anxiety-related behaviors in rodents (Pellow et al., 1985; Walf & Frye, 2007). However, the exact relation between AN and general anxiety is not clear. Although there is remarkable comorbidity of anxiety disorders and AN (Kaye et al., 2004), many patients do not display anxiety-related symptoms in non-food contexts. Possibly, AN microbiome-colonized animals in the current study did not show increased anxiety-related behaviors in EPM and OF, but would have shown increased food-specific anxiety in a conditioned taste aversion paradigm.

In vivo fiber photometry of VTA DA neurons for aberrant reward processing. No alterations of VTA DA phasic responses to sucrose reward and reward prediction (errors) by the intervention were reported here. Notably, the anatomically and functionally distinct DA subpopulations of the VTA have distinct roles in reward processing (Lammel et al., 2014) and may also respond differently to changes in gut microbiome. Using this experimental design, it is impossible to detect small-scale microbiota-induced effects on phasic responses of specific subpopulations of the VTA. Moreover, VTA DA phasic responses to reinforcement may not be altered in AN. Other aberrances of the mesolimbic pathway may underlie the aberrances in food-related reward experience and decision making. Possibly, not the VTA DA neurons, but the NAc medium spiny neurons are hyperresponsive to (food-related) reinforcers. Through an upregulation of the expression level or through sensitization of DA receptors, NAc neurons could be hypersensitive to DA, leading to aberrant reinforcement behaviors in spite of normally functioning VTA DA neurons. Other hypotheses could be that not phasic firing, but tonic firing of VTA DA neurons is altered or that the inputs to the VTA or NAc, for example from brain regions involved in cognitive control, are altered in AN. If the anorectic microbiome could induce any of these alterations, they would not have been detected with the current set-up.

In summary, intervention effectiveness has not yet been quantified, microbial strain-specific effects may be missed when analyzing microbiome effects based on donor health status only and the measured outcome parameters may have been limited in their ability to dissociate between healthy and AN phenotypes. Further analysis of the samples and data obtained in this study could in part address previously mentioned discussion points.

Determination of the microbiome composition in the stool samples of the animals is currently in the pipeline. By extracting and sequencing the bacterial DNA in the stool samples, the microbiome composition of the animals at the time of each measurement will be determined. By similar means, the taxa abundance distribution in the fecal samples of the donors will be determined and compared to verify that healthy controls differed from AN patients in taxa abundance distribution. By comparison of baseline and post-antibiotics microbiomes of antibiotics-treated animals, effectiveness of the antibiotics treatment in depleting the host microbiome can be determined. Comparing the microbiomes of the donors and the recipient rats post-transplant (AN transplant and HC transplant group) allows for the measurement of fecal transplant effectivity. Furthermore, the stability of the taxa abundance distribution in the microbiomes can be studied by comparing the microbiota in the stool samples of the relevant measurements over time per group (i.e. post-antibiotics vs post-transplant, EPM and OF measurements for the antibiotics group). Furthermore, differences between the intervention groups after antibiotics treatment and fecal transplant will be quantified by comparing the group averaged alpha diversities (number and distribution of bacterial

strains) of the microbiome. Lastly, the analysis of the microbiome composition from the stool samples allows for genera-specific correlation analyses independent of donor health status.

As the analyzed outcome parameters of reversal learning and reward processing may have been limited in their ability to show an AN phenotype, it is of interest to further explore the data. Interestingly, Geisler and colleagues reported no difference between AN patients and controls in the total amount of reversals, but did report differences in more subtle behavioral parameters, like perseverative errors and responses after a win or loss (Win-Stays, Win-Shifts and Lose-Stays, Lose-Shifts). Data reflecting these behaviors can also be extracted from our probabilistic reversal learning data set. Furthermore, it can also be of interest to look at the phasic activity of the VTA DA neurons following the lever press in these specific reversal learning behavioral events. Additionally, the signal in the seconds preceding the lever press may reflect VTA involvement in the decision making process, and could be compared between the intervention groups. Lastly, the raw F signal before the occurrence of any phasic response-triggering event can be considered a proxy for tonic VTA DA activity, which would be interesting to look into because tonic VTA DA activity may have a role in reward value encoding (Wang et al., 2021), affects striatal DA levels, in synchrony and balance with phasic firing determines striatal D1/D2 receptor levels and is altered by food restriction (Carr, 2020; Dreyer et al., 2010). However, since F is also heavily affected by the detection efficiency, that may differ between subjects and measurements, interpretation of such results should be done with care. Thus, although current analyses revealed no effect of AN-specific dysbiosis on reversal learning and VTA reward processing, further exploration of the reversal learning and photometry data may reveal effects on other measured parameters and is therefore encouraged.

In summary, this study is one of the first to investigate a causal relationship between AN-specific dysbiosis and AN phenotype development. The current findings do not support a role for AN-specific dysbiosis in cognitive inflexibility, anxiety and aberrant reward signaling. Intervention effectiveness however remains to be quantified and further exploration of the obtained data is encouraged. Given the complexity of the gut microbiome and its interaction with the brain, detecting AN-specific microbiota-induced effects is challenging. It is pivotal to establish which specific microbial strains are associated with which specific AN phenotypes in humans, before studying causality with fecal transplantation studies. Thus, although fecal transplantation studies are not yet consistent in the report of a role for AN-specific dysbiosis in AN pathophysiology, it remains of interest to study microbiota-behavior associations in AN. Large-scale correlation studies seem to be an important first step towards demystification of the complex relation between gut microbiota and AN.

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Supplement

Descriptive statistics probabilistic reversal learning task

Relative reversals		
Effect microbiome depletion with antibiotics (Fig. 2F)		
Group	*Before antibiotics*	*After antibiotics*
Antibiotics	3.7 ± 0.2	4.0 ± 0.2
No antibiotics	4.0 ± 0.5	4.6 ± 0.5
Effect microbiome transplant (Fig. 2H)		
Group	*Before transplant*	*After transplant*
AN transplant	4.1 ± 0.6	4.0 ± 0.3
HC transplant	3.6 ± 0.3	4.0 ± 0.2
Antibiotics no transplant	3.4 ± 0.3	4.1 ± 0.5
No intervention	4.0 ± 0.5	4.4 ± 0.4
Trials		
Effect microbiome depletion with antibiotics (Fig. 2F)		
Group	*Before antibiotics*	*After antibiotics*
Antibiotics	167.3 ± 12.8	176.6 ± 13.4
No antibiotics	200.6 ± 23.1	207.9 ± 24.8
Effect microbiome transplant (Fig. 2H)		
Group	*Before transplant*	*After transplant*
AN transplant	160.9 ± 24.7	265.3 ± 33.1
HC transplant	164.1 ± 20.5	262.4 ± 14.9
Antibiotics no transplant	171.3 ± 25.2	223.4 ± 42.0
No intervention	200.6 ± 23.1	274.1 ± 25.1

Table 1. Performance parameters probabilistic reversal learning task at baseline and after antibiotics treatment and microbiome transplant. *Data represent mean ± SEM.*

Descriptive statistics Go/NoGo task

Overall performance (Fig. 4A)		
Group	*Before transplant*	*After transplant*
AN transplant	57.9 ± 10.3	61.0 ± 9.7
HC transplant	57.5 ± 9.5	56.5 ± 9.6
Antibiotics no transplant	54.9 ± 2.4	45.1 ± 7.3
No intervention	54.0 ± 5.2	45.1 ± 7.3
%Correct NoGo trials (Fig. 4B)		
Group	*Before transplant*	*After transplant*
AN transplant	38.0 ± 13.9	40.0 ± 18.1
HC transplant	37.9 ± 13.9	51.8 ± 8.3
Antibiotics no transplant	34.1 ± 0.3	26.0 ± 20.0
No intervention	37.2 ± 18.1	14.6 ± 14.6
Premature lever presses (Fig. 4C)		
Group	*Before transplant*	*After transplant*
AN transplant	19.0 ± 7.8	25.7 ± 8.3
HC transplant	27.0 ± 5.8	20.7 ± 4.7
Antibiotics no transplant	27.0 ± 4.5	33.5 ± 10.5
No intervention	27.5 ± 9.0	39.0 ± 8.0
Premature head entries (Fig. 4D)		
Group	*Before transplant*	*After transplant*
AN transplant	12.3 ± 1.9	2.7 ± 0.7
HC transplant	3.0 ± 1.8	3.7 ± 2.2
Antibiotics no transplant	4.5 ± 4.5	5.5 ± 0.5
No intervention	2.8 ± 0.3	2.5 ± 0.5

Table 2. Performance parameters Go/NoGo task at baseline and after microbiome transplant. *Data represent mean ± SEM.*