Microbiota Influence on Behavior: Integrative Analysis of Serotonin Metabolism and Behavioral Profile in Germ-Free Mice

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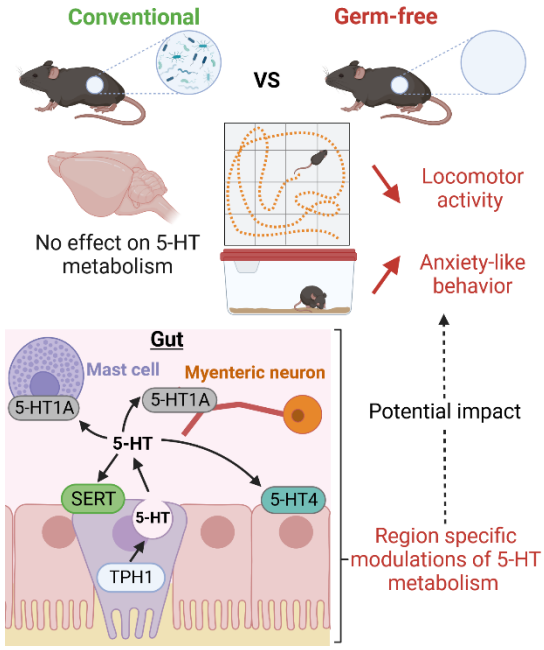
Abstract : Previous studies on germ-free (GF) animals have described altered anxiety-like and social behaviors together with dysregulations in brain serotonin (5-HT) metabolism. Alterations in circulating 5-HT levels and gut 5-HT metabolism have also been reported in GF mice. In this study, we conducted an integrative analysis of various behaviors as well as markers of 5-HT metabolism in the brain and along the GI tract of GF male mice compared to conventional (CV) ones. We found a strong decrease in locomotor activity, accompanied by some signs of increased anxiety-like behavior in GF mice compared with CV mice. Brain gene expression analysis showed no differences in HTR1A and TPH2 genes. In the gut, we found decreased TPH1 expression in the colon of GF mice, while it was increased in the cecum. HTR1A expression was dramatically decreased in the colon, while HTR4 expression was increased both in the cecum and colon of GF mice compared to CV mice. Finally, SLC6A4 expression was increased in the ileum and colon of GF mice compared to CV mice. Our results add to the evidence that the microbiota is involved in regulation of behavior, although heterogeneity among studies suggests a strong impact of genetic and environmental factors on this microbiota-mediated regulation. While no impact of GF status on brain 5-HT was observed, substantial differences in gut 5-HT metabolism were noted, with tissue-dependent results indicating a varying role of microbiota along the GI tract.



Key words: Microbiota, Brain, Intestines, Serotonin, Locomotion, Anxiety.

1 INTRODUCTION

Germ-free (GF) animals are devoid of living microorganisms and are thus good models to investigate the impact of the lack of microbiota on brain development, brain function and behavior. In the past decade, studies investigating behavior of GF rodents revealed that the absence of microbiota led to altered anxiety-like behavior, locomotor activity, social behavior and memory, when compared to conventional (CV) rodents (detailed in supplementary table S1) [1–17]. Some of these studies have also assessed various brain parameters, and, in particular, have revealed alterations in serotonin (5-HT) signaling [1,3,4,8,12,15].

5-HT plays a key role in various brain functions and behaviors. It is commonly known to be involved, for instance, in anxiety, depressive-like behaviors and memory, but there is also evidence for its role in social and repetitive behaviors [18,19]. Brain 5-HT is mostly produced by catalysis of tryptophan into 5-HT by tryptophan hydroxylase 2 (TPH2) in the raphe nuclei serotonergic neurons, which send multiple projections to the cortex, striatum, hippocampus and amygdala [20]. However, the contribution of 5-HT is not limited to the central nervous system. Indeed, ninety-five percent of circulating 5-HT is produced by the gut enterochromaffin cells (ECC) where tryptophan hydroxylase 1 (TPH1) catalyzes the conversion of tryptophan to 5-HT. In the gut, 5-HT contributes to the regulation of motility, neuronal transmission and vasoconstriction[21]. As both central and peripheral 5-HT play a role in pre- and postnatal neurodevelopment, their proper regulation is necessary for brain function and behavior [22,23].

The microbiota can modulate 5-HT metabolism in the gut. Indeed, serum 5-HT levels, and the expression of genes and proteins involved in 5-HT metabolism have been found to be dysregulated in the gut of GF or antibiotic treated rodents [24–27] (detailed in table S2). Interestingly, in some of those studies, alterations in serum 5-HT levels and gut 5-HT metabolism were reversed by colonization of GF mice with specific pathogen free (SPF) microbiota, spore forming bacteria or administration of short-chain fatty acid (SCFA) [24–26]. In addition, even if cerebral levels of 5-HT are independent of intestinal 5-HT production, there has been evidence in other studies of an impact of the intestinal microbiota on serotonergic metabolism in the brain[1,3,4,8,12,15,28] (detailed in table S2). Moreover, modulation of the microbiota by probiotic treatments has been shown to impact the expression of genes involved in 5-HT metabolism in the gut or brain in different studies [29–31].

However, no prior study has simultaneously considered both intestinal and cerebral 5-HT. For this reason, we conducted an integrative analysis of gut and brain 5-HT systems to highlight the importance of the microbiota-gut-brain axis in regulation of gut and brain 5-HT and its consequence on behavior. We compared GF C57BL/6J male mice to conventional (CV) C57BL/6J male mice. We chose this mouse strain because it is the most common background for genetically modified strains, and thus a highly used strain in research. Both GF and CV mice were housed and tested in identical isolators. We assessed anxiety-like behavior, repetitive behaviors, social behavior, and spatial memory in a battery of behavioral tests, some of which reflecting multiple behavioral traits through different parameters.

2 MATERIALS AND METHODS

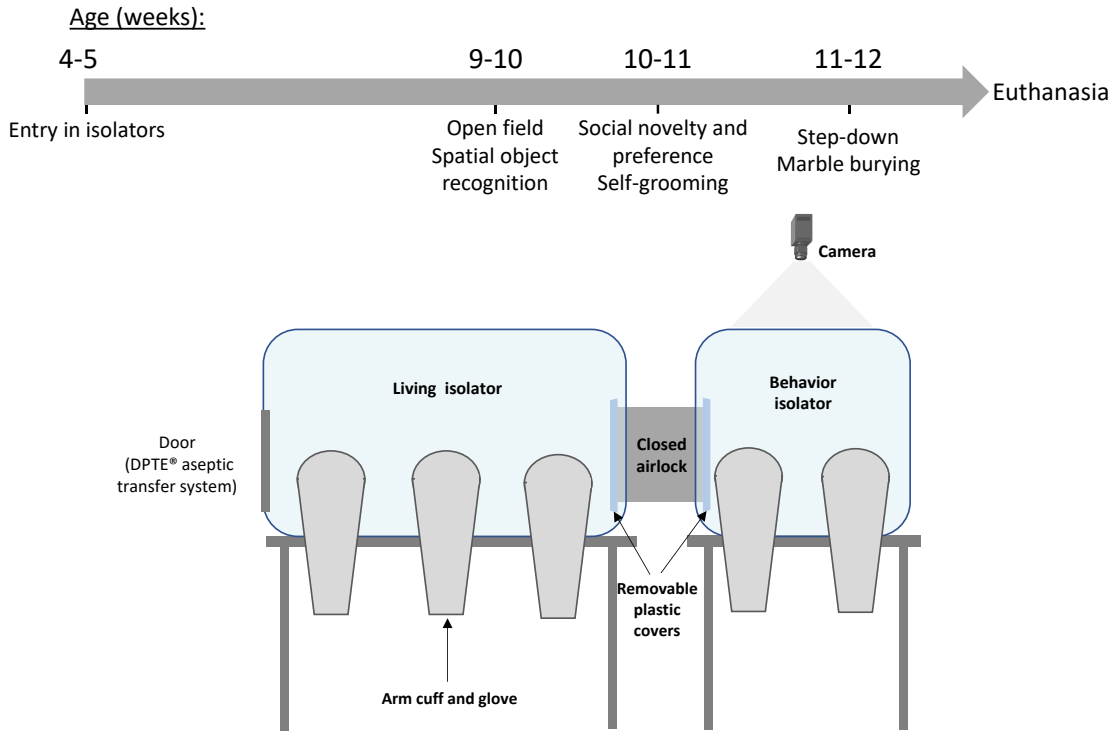


Figure 1: Calendar of the experiment and schematic representation of the isolators.

2.1 Animals

All procedures were carried out in accordance with the European guidelines for the care and use of laboratory animals and approved by the ethics committee of the INRAE Research Center at Jouy-en-Josas (approval reference: APAFIS # 22637)

Each group was composed of 15 male C57BL/6JCrl mice either GF or CV. CV mice were obtained by recolonizing GF mice with SPF microbiota and breeding them over two generations (Anaxem facility, Micalis Institute, Jouy-en-Josas, France). GF mice were born in breeding isolators, and CV mice in a conventional breeding room in the same facility. At 4-5 weeks of age, all mice were transferred according to their bacterial status to two identical experimental isolators (Piercan, Bondy, France) and housed in cages of four. Isolators of both groups were in the same room to avoid environmental bias. Each isolator was comprised of two separated compartments: a living compartment and one dedicated to behavioral testing (Fig 1). Four additional male mice of the same strain and bacterial status were also brought into the isolators at the beginning of the experiment to serve later as stranger mice for the social interaction test. Mice had access to γ-irradiated (45 kGy) standard diet (R03; Scientific Animal Food and Engineering, Augy, France) and autoclaved tap water *ad libitum.* Transparent plastic tunnel, chewing sticks and nesting material were placed is each cage for environmental enrichment. Mice were exposed to an artificial light of 100 lux (with a 12-h light/dark cycle) and a temperature between 20 and 24°C. The GF status of the animal was monitored every week by microscopic examination and aerobic and anaerobic cultures of drinking water and freshly voided fecal samples.

At the end of the experiment, animals (13 weeks old) were weighed and killed by decapitation. Their cecum was dissected and weighed full and empty. At autopsy, 0.5 cm sections of ileum, cecum and colon were put in RNA later™ (Fisher Scientific, Illkirch, France) at 4°C for 24 h and then kept at -80°C. The brain was flash frozen in isopentane and kept at -80°C before being cut into 100 µm anteroposterior coronal sections using a Leica CM3050S cryostat (Leica Biosystems, Nanterre, France). Brain sections were kept at -80°C until use.

2.2 Behavior

The mice were between 9 and 13 weeks old at the time of the behavioral tests. Behavioral tests were performed in the behavioral compartment of the isolator (Fig 1) between 9 am and 3 pm, simultaneously in the GF and CV isolators, by two experimenters who had been regularly involved in handling the mice throughout the protocol. The step-down and marble burying tests were analyzed in real-time. The open field (OF), spatial recognition, social interaction and self-grooming tests were video recorded for later analysis. All videos were analyzed using the ANY-maze software (Stoelting Co., Dublin, Ireland), either automatically (OF and spatial object recognition tests) or by manual count on the software in a blinded setting (social interaction, self-grooming and motor stereotypies assessment). For the self-grooming measures, the analysis of two separate experimenters was averaged. The animals from each group were evenly distributed between the experimenters both for handling and analysis in real-time.

2.2.1 **Anxiety-like and stereotyped behaviors:**

2.2.1.1 Open field

Adapted from [32]. The test was conducted in a dimly lit square OF of 45x45 cm and a 20 cm height with opaque walls and lasted 5 min. Mice were always placed in the bottom right corner of the OF at the start of the test. The total distance covered was measured to assess locomotor activity. The percentages of time spent, and the distance covered in the central zone, number of entries in the center and percentage of time spent in the corners were measured to assess anxiety related behaviors. It is considered that the anxiety of a mouse is inversely proportional to the time spent, the number of entries or the distance traveled in the center (anxiogenic zone), and proportional to the time spent in periphery, especially in the corners. No mice in the study met the exclusion criterion of travelling less than 2 m during the test.

2.2.1.2 Step-down test

Adapted from [33]. Mice were placed on a platform 12.5 x 9.5 cm wide and 4.0 cm high, located in the center of the behavior isolator. The latency required for the mouse to step down from the platform was measured. Once the mouse stepped down or after a maximum duration of 5 min without stepping down, the mouse was left to explore on the floor around the platform for 5 s, then returned to its cage. The test was repeated three times with a 1-min delay between each run on the platform during which the mouse was back in its home cage. A longer time spent on the platform reflects higher anxiety. The average latency to step down between the three trials was used for statistical analysis.

2.2.1.3 Self-grooming and motor stereotypies assessment

Adapted from [34,35]. Mice were placed in an empty clean cage with a small amount of bedding and filmed from the side for 10 min. Total number of grooming bouts, the percentage of incomplete grooming bouts, rearing, digging, and circling episodes were recorded as markers of repetitive behaviors (motor stereotypies). Total grooming time and the latency to first grooming were recorded as markers of anxiety-like behavior. A complete grooming bout was defined by the passage from snout/head grooming to body grooming, staying at least two seconds on each part. An incomplete grooming bout was defined as grooming only one part of the body, even if it lasted several seconds.

2.2.1.4 Marble burying test

Adapted from [36] . Mice were placed in an empty clean cage with 4 cm of clean litter mixed with some litter from the home cage, on the surface of which were placed three rows of four marbles (1.5 cm in diameter). Mice were left in the cage for 30 min, during which the number of marbles buried (defined as two thirds of the marble not visible, pictured in supplementary methods) was manually recorded every 5 min by two experimenters who each analyzed half of the animals in each group. A higher number of buried marbles reflects increased repetitive behaviors.

2.2.2 **Social interaction**

Adapted from [37]. This test also took place in the OF, in which two transparent perforated plexiglass cylinders were placed in opposite corners, 5 cm from the edge of the OF. The cylinders were 10 cm in diameter and 14.5 cm high with an opaque plexiglass cover. A second cylinder of the same diameter and 8 cm high was placed on the top of the first one to prevent the animals from climbing. The test consisted of three 5-min phases: the habituation phase with two empty cylinders, the social novelty phase with a stranger mouse in one of the cylinders and the social preference phase, with a new stranger mouse in the previously empty cylinder. Localization of the "Stranger mouse cylinder" was alternated between each test. The total distance traveled, and time spent interacting with each of the cylinders (interaction with the snout) were measured during the three phases. The interaction with the right cylinder in the habituation phase, the cylinder containing the mouse in the social novelty phase and the cylinder containing the new stranger mouse in the social preference phase were expressed as percentage of total interaction time. This percentage was compared between groups and to a theoretical value of 50 %, considering that if it was significantly higher than 50 %, mice showed a preference for this cylinder. As mice naturally tend to go towards other animals, and towards novelty, this percentage is expected to be higher than 50% for mice presenting normal social behavior. Mice that did not interact with one of the cylinders during the habituation phase were excluded from the analysis. This concerned one mouse in the GF group.

2.2.3 **Spatial memory: spatial object recognition test**

Adapted from [38]. This test was composed of five phases. The duration of each phase was 5 min separated by a 3-minute interval during which the mice returned to their home cages. Visual cues (printed sheets of contrasting colored shapes) were placed on the outside of the four walls of the isolator. The OF test was used as the first phase (P1), allowing the mice to accustom themselves to the test environment. During the next three phases (P2-P4), a Lego® plate (25 x25 cm) was placed in the center of the OF, on which were placed 5 Lego® objects (ToyPro, Nederweert, The Netherlands). The mouse was placed in the center of the OF and left to explore the environment and the objects. For phase five (P5) the position of two objects was switched (always the same two objects, referred to as "displaced objects", pictured in supplementary methods). The time the animal spent with its snout in contact with each object was measured in P4 and P5 to calculate **DO=**Average time spent interacting with objects 1 and 4 (that are the "displaced objects" in P5), and **NDO=**Average time spent interacting with objects 2,3 and 5 (that are the "non-displaced objects" in P5). We measured DO and NDO in P4 to ensure that the mice from either group had no natural preference for the future displaced objects. During P5 we measured the recognition index using the following formula: **RI= (DO - NDO)/AnyObj,** with **AnyObj=**average time spent interacting with any object. Dividing by AnyObj allows to correct for bias due to potential differences in interaction time due to mobility differences, unrelated to a preference for an object [39]. Total distance traveled was measured in both phases to assess locomotor activity. No animal met the exclusion criteria of not interacting with all objects at least once in phase 2 to 4 and/or having a total interaction time of less than 3 s in P5.

2.3 RT-qPCR

One to 2 mm circles of selected brain section areas (prefrontal cortex, nucleus accumbens, hippocampus and cerebellum) were punched out from the sections using Punches kit® (World Precision Instruments, Hessen, Germany) and kept at -80°C before use. Localization of the punches were chosen according to Paxinos and Franklin (2007) [40]. See supplementary table S3 for details of the punches. Brain punches were homogenized with a pipette in 500µL of Nucleozol (Macherey Nalgel, Düren, Germany) before total RNA extraction using the NucleoSpin® RNA XS kit (Macherey Nalgel, Düren, Germany). Gut samples were homogenized in 600 µL of lysis buffer (4 µL dithiothreitol 1 M (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and 3 µL reagent DX in 573 µL RLT Plus buffer) with a stainless-steel bead (Qiagen, Courtaboeuf, France). Homogenization of gut samples was performed at 1800 rpm for 2 min in a Powteq GT300 grinder (Grosseron, Couëron, France) using pre-colded rack. Total RNA from gut

sections were extracted using the RNeasy Plus mini kits (Qiagen, Courtaboeuf, France) following manufactory protocol. Gut sections and brain punches were eluted in a final volume of 30µL and 10µL respectively. Real-time qPCR was performed based on TaqMan gene expression assays with predesigned Taqman primers and probes for the mice (Assays-on-Demand™ , Table S4) using StepOne™ Real-Time PCR System (Applied Biosystems, Fisher Scientific, Illkirch, France) (2 min at 50°C; 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C). Cycle threshold (CT) were normalized by subtracting CT of the housekeeping gene (β-actin). Then 2eΔΔCT were calculated using the CV group for normalization and used for statistical analysis.

2.4 Statistical analysis

Calculations were performed with the GraphPad Prism software (version 7.03, La Jolla, CA, USA). Outliers (maximum one per group) were identified using the "Identify outliers" function with a Grubb value of 0.05 and removed from the statistical analysis. For tests in which datasets for all parameters followed a normal distribution and had equal group variances, GF and CV groups were compared using a Student's *t* test and data was expressed as means ± standard errors of the mean. If that was not the case for at least one parameter of the test, all parameters were analyzed with a Mann-Whitney test and expressed as medians. For the social interaction test, aside from group comparison, group medians were also compared to a theoretical value of 50 using the Wilcoxon signed rank test. For the step down test, an additional Khi-2 contingency test was used, considering "over 30 s latency" and "under 30 s latency" as two categories. For tests where the distance traveled could be measured, Spearman's correlations were calculated between this parameter and the other behavioral parameters to assess if they were likely to be influenced by the animal's locomotor activity. For tests where distance could not be recorded, an average distance from all the other tests was used. For all statistical tests, the level of significance was set at $p < 0.05$.

3 RESULTS

3.1 GF mice show strongly reduced locomotion compared to CV mice.

We measured the total distance traveled during the OF test. We also measured it during social interaction and spatial object recognition tests and, within each of those tests, we averaged the distances traveled during the different phases. In all tests, GF mice consistently showed a greatly decreased locomotor activity compared to CV mice (OF: $p<0.0001$; Social interaction: $p=0.0003$; Spatial object recognition: $p=0.0001$) (Fig 2).

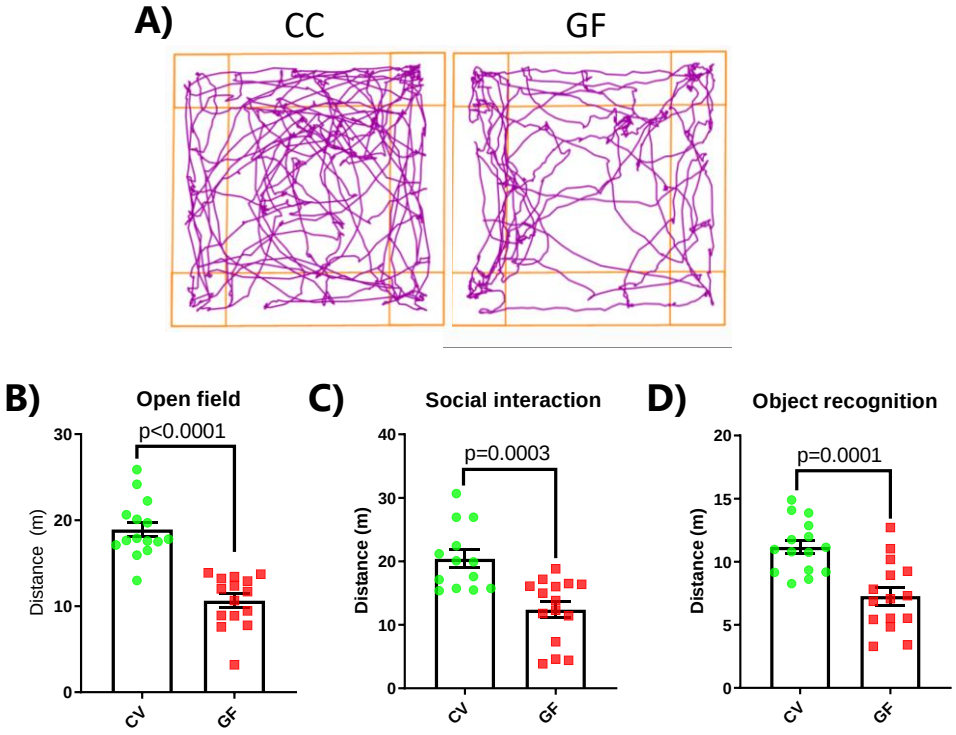


Figure 2 : Locomotor activity of CV and GF mice. A) Representative track plots of the OF test **B)** Total distance traveled during OF test **C)** Average total distance traveled during all three phases of the social interaction test. **D)** Average total distance traveled during phases 4 and 5 of the object recognition test. No outliers.

3.2 GF mice show some signs of increased anxiety like behavior.

In the OF test, GF mice entered the central zone less often ($p=0.003$) (Fig 3 C) but there was no significant difference in the percentage of distance traveled, or percentage of time spent near the center of the OF (Fig 3 A, B). There was also no difference in the percentage of time spent in the corners of the OF (Fig 3 D). The decreased number of entries into the central zone observed in GF mice is likely in great part due to their decreased locomotor activity compared to CV mice. Indeed, Spearman's correlation analysis revealed a strong positive correlation between the number of entries into the center zone and total distance traveled during the test ($R^2=0.7$, $p<0.0001$). No other parameter from this test was significantly correlated to distance traveled.

During the step-down test, the latency to step down was not significantly different between the two groups (Fig 3 E). However, a third of GF mice had a latency to step down superior to 30 s while none of the CV mice did; a Khi² test comparing the proportion of mice in each group with a latency to step

down over or under 30 s revealed a significant difference between the CV and GF mice ($p<0.0006$) (Fig 3 F). In the self-grooming test, GF mice showed an increased grooming time ($p=0.02$), and a decreased latency to first grooming ($p=0.002$) when compared to CV mice (Fig 3 G-H) indicating an increased anxiety in the GF group. A Spearman's correlation analysis revealed that the distance traveled (in average during all tests in which it was measured) was negatively, albeit weakly, correlated with the total grooming time ($R^2=-0.39$, $p=0.04$). Thus, the locomotion difference between groups seems to have only a weak effect on this parameter. There was no significant correlation between distance and latency to first grooming or latency to step down, indicating that the locomotion difference did not impact those parameters.

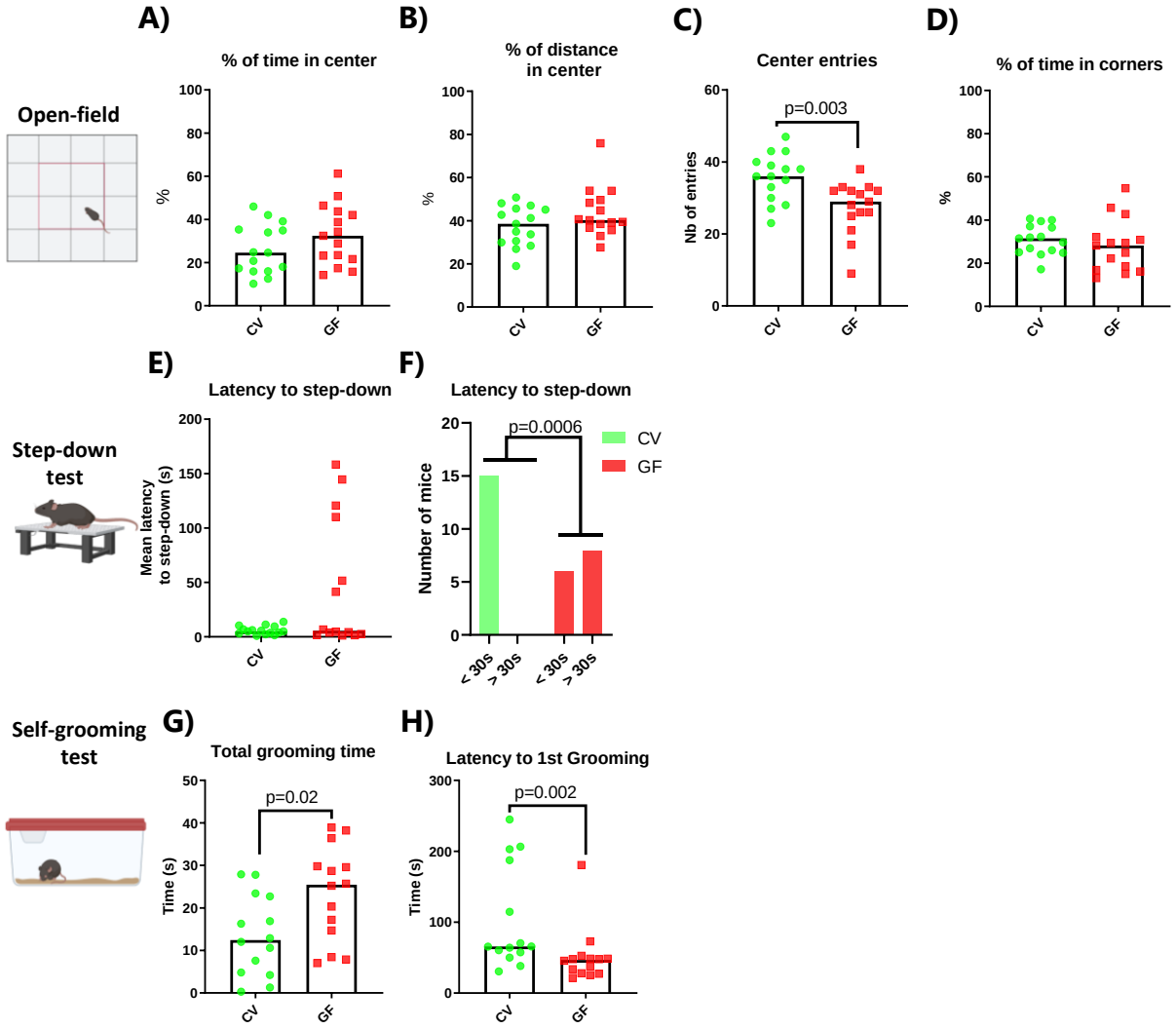


Figure 3 : Anxiety related behavioral parameters in CV and GF mice during OF test (A-D), step down test (E-F) and self-grooming test (G-H). No outliers. Schematics created with Biorender.com.

3.3 GF status does not affect repetitive behaviors.

During the self-grooming test, there were no differences between groups in the total number of grooming bouts, percentage of incomplete grooming (Fig 4 A-B) or other motor stereotypies such as digging and circling (Fig 4 C-D). However, GF mice displayed decreased rearing behavior during the test ($p<0.0001$) (Fig 4 E), likely influenced in part by their decreased locomotor activity. Indeed, Spearman's correlation analysis revealed a significant positive correlation between distance traveled and the number of rearing ($R^2=0.56$; $p=0.001$). In the marble burying test, the number of marbles buried was not different between groups (Fig 4 F). There was no other significant correlation between distance and the parameters

included in Fig 4. Overall, GF status did not seem to influence repetitive behaviors in this study.

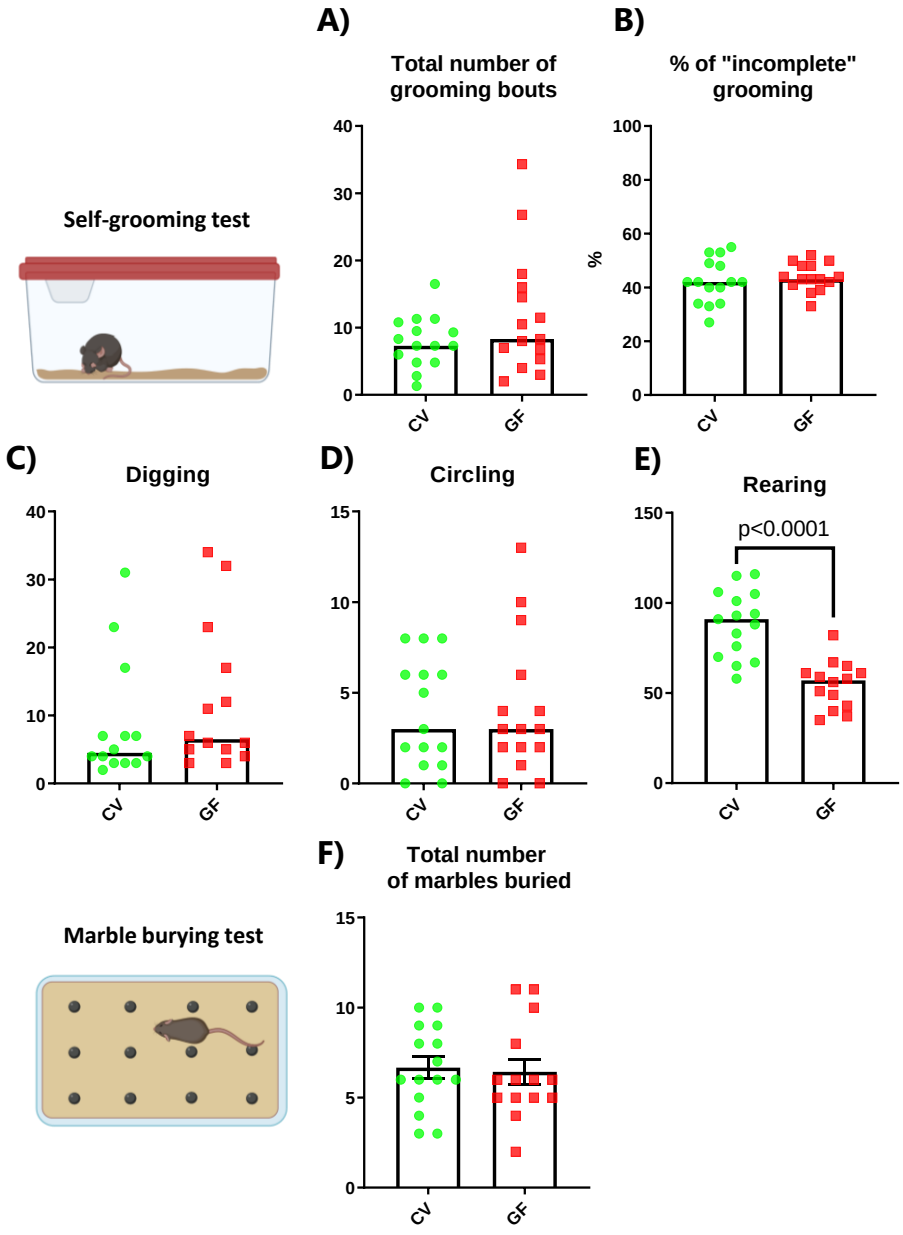


Figure 4 : Repetitive behaviors in CV and GF mice. A-B) Self-grooming stereotypies **C-E)** Other motor stereotypies during self-grooming test **G)** Marble burying test. In parameters illustrated in figures A), B) and C), one outlier was removed in each group. In parameter illustrated in fig E), one outlier was removed in the GF group. Schematics created with Biorender.com.

3.4 GF mice show a trend to enhanced sociability.

GF and CV mice had no preference for any cylinder during the habituation test (Fig 5 A), but a strong preference for the cylinder containing the stranger mouse in the social novelty phase, as both groups spent significantly more than 50 % of the interaction time with the “Stranger mouse cylinder” (CV p=0.0002 GF p=0.0001) (Fig 5 B). The percentage of interaction with this cylinder was not significantly different between groups, although there was a trend for a higher percentage of interaction with the stranger mouse cylinder in the GF group (p=0.06). For social preference, only the GF mice spent significantly more than 50 % of the interaction time interacting with the “New stranger mouse cylinder” (p=0.02) (Fig 5 C) but there were no significant differences between groups in the percentage of

interaction with this cylinder. There were no significant correlations between average distance traveled during the three phases of the test and any of the percentages of interaction.

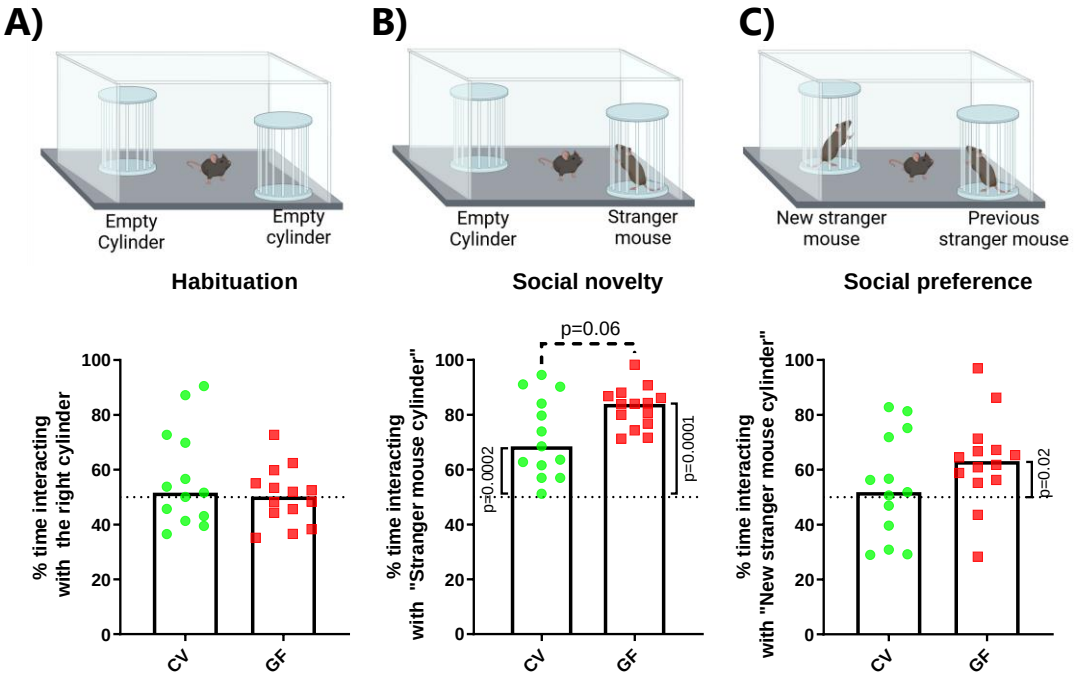


Figure 5 : Performance of CV and GF mice in social novelty and preference tests. A) Habituation phase **B)** Social novelty phase **C)** Social preference phase. No outliers. Schematic created with Biorender.com.

3.5 GF status does not affect spatial object recognition memory.

In phase 4 of the object recognition test, we confirmed that mice from either group showed no preference for the future displaced or non-displaced objects by comparing DO and NDO in each group (CV: Mean DO= 5,85 Mean NDO=5,54 p=0.77; GF: Mean DO=2.43 Mean NDO= 3.10 p=0.61; data not shown). In P5, we measured the recognition index as detailed in the materials and methods and found no significant difference between groups (Fig 6). There was no significant correlation between average distance traveled during P4 and P5 and the recognition index.

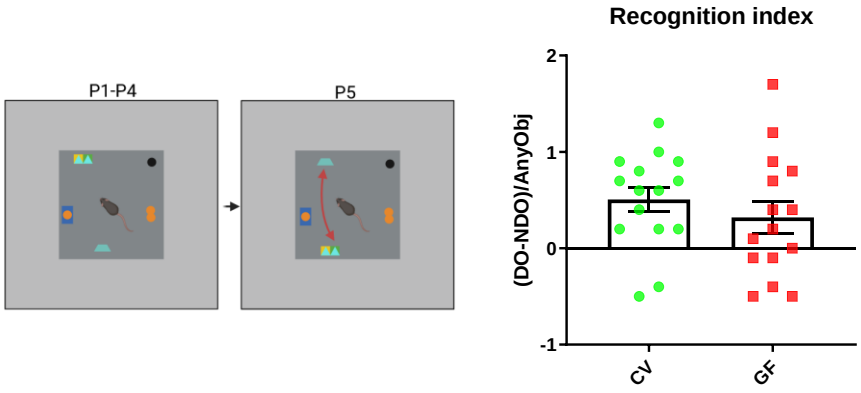


Figure 6 : Performance of CV and GF mice in the spatial object recognition test. No outliers. Schematic created with Biorender.com

3.6 GF mice do not display differences in expression of TPH2 and HTR1A in the brain.

We measured relative gene expression of TPH2 and HTR1A (coding for the 5-HT1A receptor) in the hippocampus, prefrontal cortex, nucleus accumbens and cerebellum by RT-qPCR. There were no significant differences between GF and CV mice in the expression level of either of those genes (Fig S1).

3.7 Increased weight of cecal content and mucosa in GF mice

GF mice had very significantly enlarged caeca, characterized by an increased weight of cecal content ($p<0.0001$) and cecal wall ($p<0.0001$) (Fig 7 C-D) compared to CV. This was expected as it is a characteristic of GF mice [41]. The body weight was increased in GF mice compared to CV ($p=0.005$) (Fig 7 A), but this difference was due to the extra weight of the cecal content, as it was no longer observed when cecal content weight was removed from body weight (Fig 7 B).

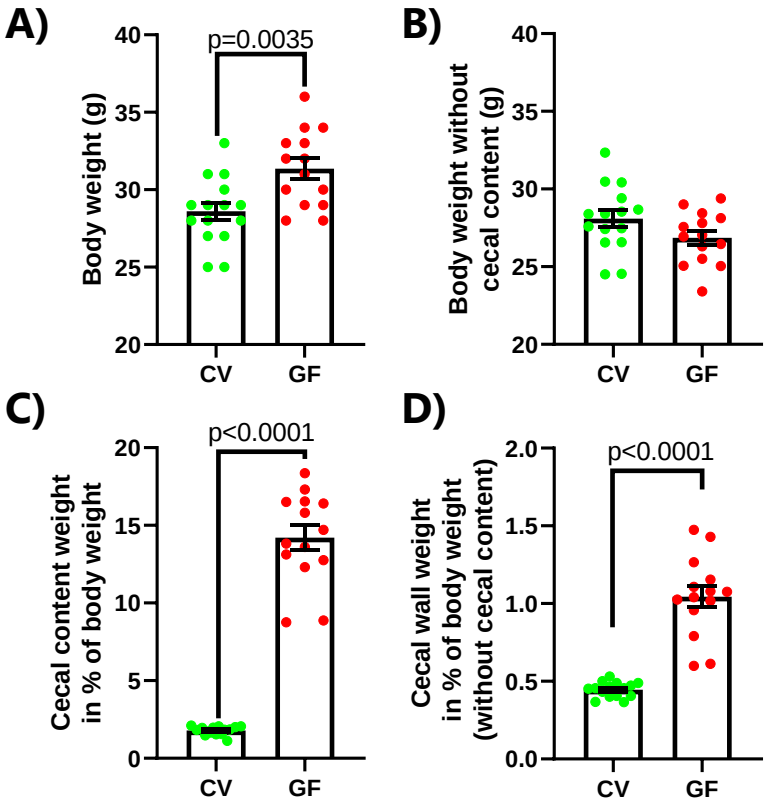


Figure 7 : Body weight and cecal weight of CV and GF mice. **A)** Total body weight **B)** Body weight without cecal content **C)** Relative weight of cecal content **D)** Relative weight of cecal wall. No outliers.

3.8 GF mice show region specific alterations in the expression of genes involved in 5-HT metabolism in the gut.

We measured gene expression of various genes involved in 5-HT metabolism in the ileum, cecum and colon of CV and GF mice. Interestingly, results varied depending on the gut segment. In the colon, there

was a 3-fold increase ($p<0.0001$) of TPH1 expression in GF mice compared to CV, while the opposite (2-fold decrease; $p<0.0001$) was observed in cecal tissue (Fig 8 A). The expression level of the HTR1A gene was dramatically decreased (20-fold decrease; $p<0.0001$) in the colon of GF mice but not in the other gut segments (Fig 8 B). The expression level of the HTR4 gene, coding for the 5-HT4 receptor, was slightly increased in the colon (1.2-fold increase; $p=0.03$) and to a greater extent (2-fold increase; $p<0.0001$) in the cecum of GF mice compared to CV (Fig 8 C). Finally, the expression of the SLC6A4 gene, coding for the serotonin transporter SERT, was slightly increased in the ileum (1.5-fold increase; $p=0.003$) and to a greater extent (3-fold increase; $p<0.0001$) in the cecum of GF mice compared to CV (Fig 8 D).

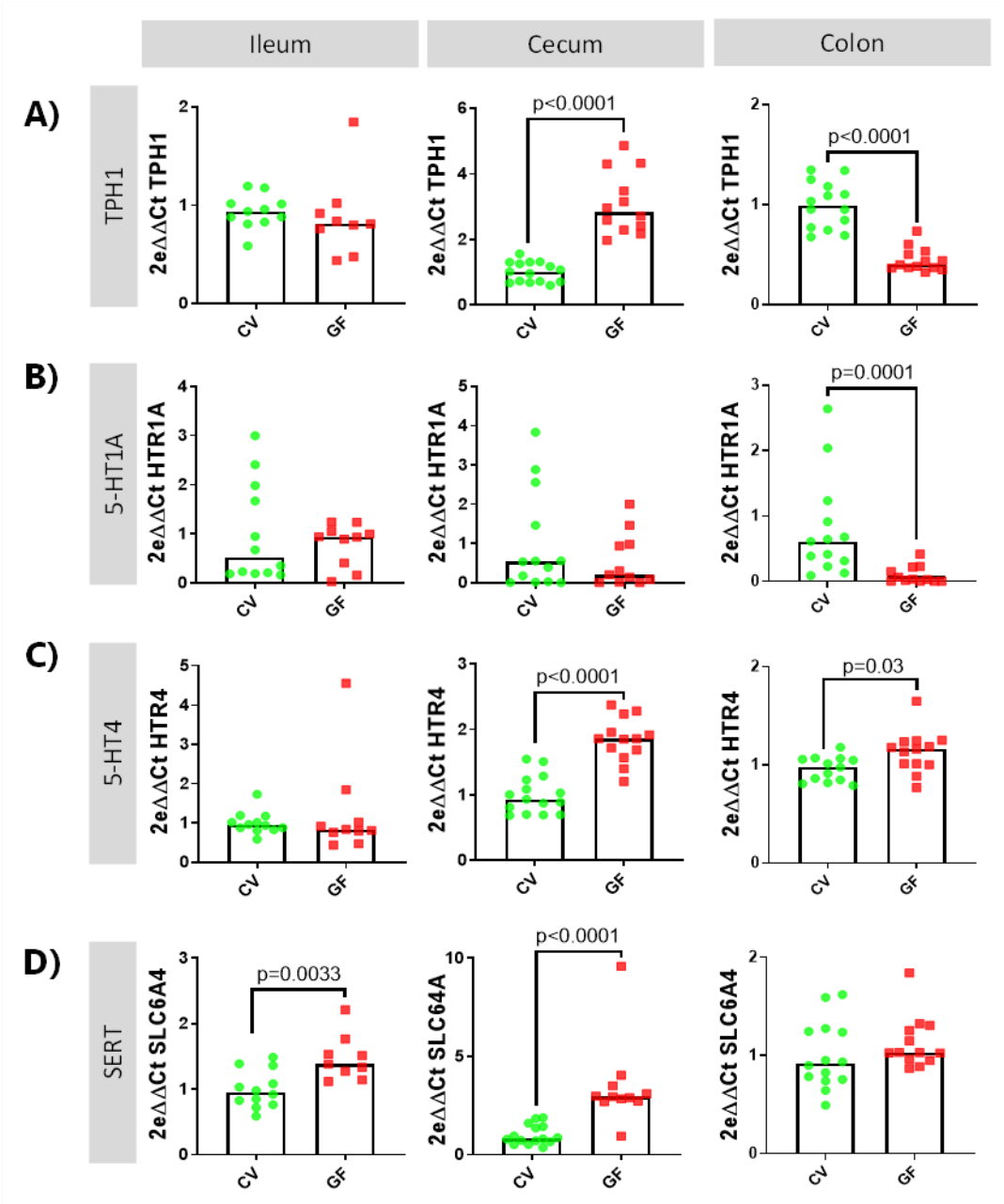


Figure 8 : Relative expression of genes involved in serotonin signaling in ileum, cecum and colon of GF and CV mice. Expressed as 2eΔΔCT of target gene – housekeeping gene (ß-actin). A) Ileum: one outlier removed in each group, Cecum: one outlier removed in GF group B) Colon: one outlier removed in each group, C) Colon: one outlier removed in CV group, D) Ileum: one outlier removed in GF group, Cecum: one outlier removed in GF group.

4 DISCUSSION

Over the years, several studies provided evidence of the implication of the gut microbiota in brain and behaviors. Our study adds to this evidence. In addition, while most studies focused on one or two behavioral outcomes (See table S1), we investigated a large array of behaviors in the same study. We confirmed some previous findings, such as increased anxiety-like behaviors and decreased locomotor activity in C57BL/6J GF mice, while also reporting on some differences. Notably, we did not observe an impaired social behavior in GF mice, despite it being a behavioral trait that has been described in GF mice of this strain[5,7,11]. We also did not observe impaired spatial memory in GF mice , while this was described by Lu et al. (2018)[5]. The behavioral alterations we observed were not accompanied by changes in expression of genes involved in the 5-HT metabolism in the brain, which differ from some observations in literature (See Table S2). However, there were strong differences between GF and CV mice in the expression of those genes in the gut. Those alterations could be associated with the behavioral changes, as 5-HT modulations in the gut could influence brain and behavior through influencing vagal transmission or the immune system[42]. This is a new finding, as no previous study investigated in parallel, behavior and brain and gut 5-HT metabolism the same animals.

Our observation of reduced locomotor activity in C57BL/6J GF mice confirms what has been observed in literature in GF and antibiotic depleted mice of this strain [5,7,43–45]. However, this is not a common characteristic of all GF mice, since other GF strains (BALB/c, NMRI and Swiss Webster) display increased locomotor activity compared to CV mice [3,6,8,9,15]. Plus, a study demonstrated that the effect of antibiotic treatment on spontaneous exercise in a running wheel was different in ICR mice bred for their "high-runner" profile, compared to normal ICR mice. This suggests that the microbiota is interacting with the genetic background to regulate locomotor activity [46]. Interestingly, GF or antibiotic treated C57BL/6J mice have dysregulated muscle glucose metabolism and mitochondrial activity compared to CV [43–45]. In one of those studies, recolonization of GF mice with SPF microbiota or SCFAs administration restored normal muscle volume and function, and increased locomotor activity and strength. Finally, other studies have shown that probiotic treatments could influence skeletal muscle metabolism and athletic performance in mice and humans [47]. Because of this, it is not surprising to see such pronounced effect of the GF status on locomotor activity in our animals. In the future, it would be interesting to carry a more in-depth analysis of motor function in GF mice and assess finer motor skills.

In our study, GF and CV mice did not show significant differences in anxiety related parameters in the OF test. In contrast, the decreased latency to first grooming and increased grooming time in GF mice compared to CV mice, as well as the greater proportion of GF mice with a latency to step down over 30 s, suggest higher anxiety-like behavior in those mice. Increased anxiety-like behavior has been previously observed in GF F344 rats, BALB/c, C57BL/6N and C57BL/6J mice compared to CV animals [4,5,8], but the opposite observation was also made in NMRI, Swiss Webster, BALB/c and C57BL/6J GF mice [1,3,6,7,9,10,12,16]. It is important to note that the choice of behavioral tests can impact the interpretation of behavioral differences. Indeed, in De Palma et al. (2015), GF C57BL/6N mice were more anxious than CV mice in the step-down test, but less anxious in the light/dark box test[16].

Moreover, it is possible that some of the discrepancies between studies are due to experimental differences. Indeed, behavioral experiments can be highly influenced by environmental factors and, in some studies, CV mice were not housed in isolators [1,5–7,11–17,48]. This could lead to behavioral differences due to the fact that GF mice would experience a more drastic environmental change than CV mice, when getting outside the isolators before the tests. By housing the control group from birth or early age in isolators, as was done in a few studies, [3,4,8–10] this issue is limited, as all mice experienced a similar environmental change before the tests. Similarly, performing the tests in isolators, as was done by Nishino et al. (2013) [8] and in our study, avoids the potential impact of early microbial recolonization on behavior. Indeed, Nishino et al. (2013), showed that GF mice that had been put outside of the isolator for 24 h showed decreased anxiety in the OF and a decreased number of buried marbles in the marble burying test, compared to GF mice that had not been out of the isolator [8].

We assessed stereotyped behaviors using the marble burying test and quantification of grooming bouts, specifically incomplete ones, as well as other motor stereotypies such as rearing, circling or digging. The only significant difference observed was a decreased number of rearing, which was likely due to the decreased locomotor activity of GF mice. Overall, this suggests that the microbiota has no effect on those stereotypies, which is coherent with what was observed in two studies in other GF strains [8,9]. However, one study observed a decreased number of marbles buried by Swiss Webster GF mice compared to CV during the marble burying test [29].

When we investigated social behavior through the social preference and novelty tests, there were no significant differences between groups, although GF mice displayed a trend to improved social behavior in the social novelty phase and had a preference for the newly added stranger mouse in the social preference phase, contrarily to CV mice. In literature, GF mice (Swiss Webster, and C57BL/6J) and GF F344 rats have been described as having impaired social behavior compared to CV animals [4,5,7,10,11,13]. However, in one study in Swiss Webster mice, GF mice had higher performances than CV mice in the three-chamber interaction test [9]. The fact that the CV mice were not housed in isolators in some studies [5,7,11,13], and that the tests were performed outside of the isolators, may partially influence the deficits in social behavior observed in GF mice, especially for social preference. Indeed, this means that the GF mice were exposed to different environmental smells during the test than they were used to in the isolators, and this may have interfered with their ability to recognize the smell of a stranger mouse. One other notable difference between literature and our study is that the stranger mice used for social novelty and preference tests were of the same microbial status as the tested animals. This was not the case in any of the studies previously mentioned that reported differences in those tests, since all used CV mice as stranger mice for both GF and CV groups [5,7,9–11,13]. It is possible that GF mice have no issues recognizing the smell or discerning between smells of other GF mice but cannot as efficiently recognize or discern between the smells of CV mice. This could explain the deficiency in social behavior observed in those studies, particularly for social preference. Discerning between smells in a complex environment could be more difficult for GF mice, as they present anatomical and functional differences in the olfactory epithelium, leading to different olfactory preferences, even for non-social odors [49–51]. However, this does not explain why the CV mice had no preference for the stranger mouse in the social preference phase of our study.

In the spatial object recognition test, we found no difference in the recognition index in GF mice compared to CV, indicating no alteration of spatial memory. This differs from the observation of impaired spatial memory in C57BL/6J GF mice in one study, albeit in a different test (Morris water maze) [5]. In addition, two other studies reported impaired non-spatial object recognition memory in GF mice compared to CV [10,52].

Overall, some of the behavioral alterations we observed between GF and CV mice differ from those described in the literature on the subject. Aside from the impact of strain and sex differences or environmental conditions on behavior, some of those discrepancies could be due to the choice of control group. Indeed, in some of the studies cited in table S1, the GF mice were from a local facility while CV animals were from a different external supplier, and sometimes only arrived in the experimental facility a few days before testing. We believe this could impact behavior, because of potential genetic differences between those animals, and potential differences in handling and living conditions in those different facilities. In this study, we tried to avoid those biases by creating a CV line in our local facility, colonizing GF mice with SPF microbiota and breeding them for two generations. Thus, our CV and GF mice have similar genetic backgrounds, were born in the same facility and have lived in an identical environment for seven weeks prior to the tests. A similar approach was used in two previous studies on one generation [8,17].

Next, we wondered if those behavioral differences were associated with changes in the expression of genes involved in the metabolism of 5-HT in the brain. For this reason, we assessed expression of genes involved in 5-HT metabolism in brain regions where 5-HT is known to play a role in the behaviors studied. However, there were no differences between groups in the expression of the TPH2 and HTR1A genes in the four brain regions assessed. A previous study has found decreased HTR1A expression in

the hippocampus of GF Swiss Webster mice compared to CV mice [1], although in another study on the same strain, no difference in the expression of this gene was found [12]. Other studies have found decreased or increased quantities of 5-HT in various brain regions of GF mice or rats compared to CV, suggesting that the lack of microbiota does impact 5-HT metabolism in the brain, albeit in different ways depending on the strain and study [3,4,8,12,15]. Assessing other actors of the 5-HT metabolism, such as 5-HT derivatives, or other receptor subtypes could have yielded different results. Nonetheless, none of those studies were performed on C57BL/6J mice, which could also explain this difference. Interestingly, a study on C57BL/6N mice did not find a significant difference in 5-HT quantity in the hippocampus between GF and CV mice [16].

As the role of 5-HT is not limited to the central nervous system, we also measured the expression levels of genes involved in 5-HT metabolism in the gut. As mentioned in the introduction, 95% of circulating 5-HT is produced in the gut by ECC, through the action of TPH1. 5-HT then plays a role in various gut functions through binding to different subtypes of 5-HT receptors, which are targets of drugs used to alleviate symptoms of various GI disorders [21]. A few studies have shown that GF mice display decreased serum and colonic levels of 5-HT compared to CV mice, which could be increased by recolonization with SPF microbiota or spore forming bacteria, or through SCFA administration [24–26,53]. While we did not measure 5-HT production directly, we investigated the expression of genes involved in 5-HT metabolism, in the colon, cecum, and ileum of the animals. In the colon, we found decreased TPH1 expression in GF mice compared to CV mice. This had also been observed in C57BL/6J and Swiss Webster mice in two studies [24,26]. In line with the decreased TPH1 expression, we also found a greatly reduced expression of HTR1A, a gene coding for the 5-HT1A receptor, in GF mice compared to CV. This receptor is expressed in myenteric neurons, where it plays an inhibitory role on neuronal transmission [54] . Thus, changes in its expression levels could impact the functioning of the enteric nervous system. It is also the main receptor involved in the response of mast cells to 5-HT, leading to their proliferation. Plus, antagonists of this receptor can limit cytokine production from T cells. Thus, this receptor could be involved in the gut immune system regulation and at interplay with the microbiota [55,56]. It is also interesting to note that GF mice display a reduced number of mast cells in the gut, with low maturation status and altered functionality leading to reduced sensitivity to allergy. Mast cells are important actors of immune homeostasis in the gut and are believed to be involved in the effect of the gut microbiota on the immune system [57] . It is possible that the disruption of 5-HT metabolism in GF mice is involved in those immune alterations.

We also found a slightly increased expression of HTR4, a gene coding for 5-HT4 receptor in the colon of GF mice compared to CV and to a greater extent in the cecum. A study on Swiss Webster mice did not see changes in the expression of this gene in the colon of GF mice compared to CV [24]. HTR4 is widely expressed along the GI tract in epithelial cells and is one of the targets of drugs that aim to regulate gut 5-HT metabolism in GI disorders. Activation of this receptor leads to increased gut motility by contraction of gut smooth muscles [21,58]. Plus, a study showed that this receptor could be activated by tryptamine, a bacterial metabolite, making it a possibly important target for an impact of the microbiota on 5-HT signaling in the gut [59].

Aside from this result, there were some differences between our observations in the colon and in the cecum and ileum. Indeed, in the cecum, we found an increased expression of TPH1 in GF mice compared to CV, contrarily to what we observed in the colon. Plus, we observed increased expression of the gene coding for SERT, SLC6A4, in the ileum and cecum, but not in the colon, of GF mice compared to CV. Increased SLC6A4 expression has been previously observed in the colon of GF mice [26] but to our knowledge no previous study has measured it in other gut segments. In the cecum, this increase is in line with the increased TPH1 expression, as there would be a need for an increased expression of SERT to regulate increased extracellular 5-HT levels.

Overall, GF mice present differences in the expression of genes involved in 5-HT metabolism with some variability depending on the gut segment studied. It is possible that the effect of microbiota on 5-HT

production is different in different gut segments. While ECCs are present all along the GI tract, the stimuli responsible for 5-HT release as well as their morphology vary depending on the gut segment observed [60,61]. Plus, some enteroendocrine cells (EEC) produce 5-HT alongside other peptides or hormones, which differ depending on the gut segment. For example, in the ileum and cecum, some EEC produce both 5-HT and cholecystokinin, while in the colon they produce both 5-HT, glucagon-like peptide 1 and PYY [61,62]. Those differences are likely to be linked to functional differences, which would require differences in regulation of 5-HT production in different gut segments. As previously mentioned, some bacterial species or SCFAs can modulate 5-HT release and TPH1 and SLC6A4 expression [24,26]. Since the composition of the microbiota greatly varies along the GI tract in mice [63], its impact on gene expression and 5-HT metabolism as a whole is likely to be different as well. This is corroborated by the fact that the decreased 5-HT observed in the colon of GF mice in two studies was not found in the small intestine and/or in the cecum [26,53]. Plus, in another study, microbiota modulation through probiotic treatments in mice affected expression of SLC6A4, TPH1 and HTR4 in different ways in ileum, cecum and colon [64]. In general, the cecum is often omitted from research papers, possibly because, while it is prominent in rodents, it does not play an important role in human gut physiology. However, in rodents, the cecum is the region where the gut microbiota is the most diverse and metabolically active along the GI tract. Thus, it is the compartment where the microbiota is most likely to strongly affect gut homeostasis and metabolism. This is in part what we observed in this study, as the difference of HTR4 and SLC6A4 gene expression between CV and GF mice was more pronounced in this compartment than in ileum or colon. For those reasons we suggest that the cecum should not be excluded from future studies investigating the impact of microbiota on gut physiology in rodent models.

As previously detailed, gut 5-HT plays a crucial role locally in the ENS, but it could have an indirect effect on brain function and behavior through its action on the immune system and the vagus nerve [42]. Thus, the behavioral alterations we observed could be in part linked to the changes in gut 5-HT metabolism. However, a more targeted investigation of the relationship between gut and brain neurotransmitters and consequences on behavior in the context of the microbiota-gut-brain axis would be necessary to investigate this hypothesis.

5 CONCLUSION:

This study adds to the evidence of an impact of the gut microbiota on brain and behavior, but also on gut physiology. We showed that the lack of microbiota strongly impairs locomotor activity in C57BL/6J male mice and induces some signs of increased anxiety-like behavior. Plus, GF mice displayed a trend to slightly increased sociability. However, the absence of gut microbiota did not seem to impact stereotyped behaviors or spatial memory. In addition, we found that the lack of microbiota induced important changes in the expression of genes involved in 5-HT metabolism in the gut, with some disparity between gut segments.

While some of those observations have been made in previous studies, there is some heterogeneity between studies with different strains or experimental conditions, suggesting that the influence of intestinal microbiota on gut, brain and behavior is complex and heavily influenced by genetic background and environmental factors. In future studies, it would be interesting to pursue in a most systematic way behavioral characterization of GF mice from different strains in the same environment. This would provide a solid knowledge basis on the behavioral profile of rodent models used in studies of the microbiota-gut-brain axis. In addition, as the gut is at the interface between intestinal microbiota and the central nervous system, it should be taken into account more systematically in future studies evaluating the influence of the gut microbiota on brain function and behavioral responses.

6 LIMITATIONS:

In this study, only adult male mice were used, which did not allow to investigate potential sex-differences, or differences due to developmental stage in the impact of the microbiota on behavior and 5-HT metabolism.

We only measured gene expression, which is a less direct expression of potential functional differences than measuring protein levels or carrying immunohistochemical analyses.

7 DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the repository of the GEMMA project at https://repository.gemma-project.eu.

8 CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

9 AUTHORS CONTRIBUTION

Léa Roussin, Laurent Naudon and Sylvie Rabot conceived and designed the research. All authors performed the research, acquired data, and participated in its analysis and interpretation. Léa Roussin, Laurent Naudon and Sylvie Rabot conceived the original draft, which was then reviewed by all the authors. Laurent Naudon and Sylvie Rabot should be considered joint senior authors.

10 ACKNOWLEDGMENTS:

We thank Sandra Letort, Victor Gomez, and Viviana Bisceglia for their work on this project as part as their undergraduate internships. We thank the Anaxem animal facility for providing the animals, equipment, and supporting staff necessary for this project.

11 FUNDING :

This study, including the PhD grant to Léa Roussin, was funded by the European Commission through the Horizon 2020 program (H2020-SC1-BHC-03-2018) as part of the GEMMA project (doi 10.3030/825033; grant number 00002771-13001252).

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13 SUPPLEMENTARY MATERIALS:

13.1 Supplementary Data :

Animals	Age and sex	CV in isolators?	Same supplier?	**Mobility**	**Anxiety**	**Repetitive behavior**	**Social behavior**	**Memory**	**Fear memory**	**Resignation**	Reference
F344 rat	M,11W	Yes	No	→	↗	N/A	↘	N/A	N/A	N/A	*1*
NMRI mice	M, 8-10W	Yes	Yes	↗	↘	N/A	N/A	N/A	N/A	N/A	*2*
Swiss Webster mice	F, 7,11W	No	No	N/A	→	N/A	N/A	↘	N/A	N/A	*3*
	F, 8W	No	Yes	N/A	↘	N/A	N/A	N/A	N/A	N/A	*4*
	F and M, 6-9W	No	Yes	N/A	↘	N/A	N/A	N/A	N/A	N/A	*5*
	M, 12W	Yes	Yes	↗	↘	→	↗	N/A	N/A	N/A	*6*
	M, 8W	No	Yes	N/A	N/A	N/A	↘	N/A	N/A	N/A	*7*
	F-M, 6-7W	Yes	Yes	↗	↘	N/A	↘	↘	N/A	N/A	*8*
	F-M 6-9W	No	?	N/A	N/A	↘	N/A	N/A	N/A	N/A	*9*
Kunming mice	M, 6W	No	Yes	→	↘	N/A	N/A	N/A	N/A	N/A	*10*
BALB/c mice	M, 7,10,16W	Yes	Yes	↗	↗	→	N/A	N/A	N/A	N/A	*11*
	M,8 W	No	Yes	↗	↘	N/A	N/A	N/A	N/A	↘	*12*
	M, 7W	No?	Yes	↗	↘	N/A	N/A	N/A	N/A	↘	*13*
C57BL/6N	M, 8-9 W	No	Yes	N/A	↗ or ↘	N/A	N/A	N/A	N/A	↘	*14*
C57BL/6J	M, 7,16W	No	No	N/A	N/A	N/A	N/A	N/A	↘	N/A	*15*
	M, 9W	No	Yes	N/A	N/A	N/A	N/A	N/A	↘	N/A	*16*
	F-M, 7-12W	No	No	→	N/A	N/A	↘	N/A	N/A	N/A	*17*
	F-M, 4,12W	No	No	↘	↗	N/A	↘	↘	↘	N/A	*18*
	M,6-8W	No	Yes	↘	N/A	N/A	N/A	N/A	N/A	N/A	*19*
	M,11-15W	No	No	↘	↘	N/A	↘	N/A	N/A	N/A	*20*
	M,11W	Yes	Yes	↘	↗	→	(↗)	→	N/A	N/A	Our study

Table S1 : Summary of the findings in literature and our study for behavior alterations in GF vs CV mice. Age indicated is at the time of behavioral tests (when precised). Some experimental differences are detailed (CV mice housed in isolators or not and use of CV and GF mice from the same supplier or not). Legend: → =No change in behavior; ↗=Behavior increased or improved in GF mice compared to CV; ↘=Behavior decreased or impaired in GF mice compared to CV; (↗)=statistical trend to an improvement in behavior in GF ; N/A= behavior not tested in this study.

Type of study	Model	Systemic and gut 5-HT	5-HT in brain	Reference
GF vs CV	F344 rats (M 11 weeks)	N/A	↘5-HT in hippocampus	*1*
	NMRI mice (M, 8 weeks)	N/A	↗5-HT turnover in striatum	*2*
	Swiss Webster mice (F, 8 weeks)	N/A	↘5-HT1A expression in hippocampus (DG)	*4*
	Swiss Webster mice (F and M, 6-9 weeks)	N/A	↗Hippocampal 5-HT, 5-HIAA, and ↘SERT expression	*5*
	Swiss Webster mice (F and M, 8 weeks)	↘ colonic TPH1 expression ↘ colonic 5-HT	N/A	*21*
	BALB/c mice (M 16 weeks)	N/A	↘5-HT turnover in striatum ↗5-HIAA in brainstem	*11*
	BALB/c mice (M, 5-6 weeks)	N/A	Upregulation of 5-HTR7,1f, 3B in hippocampus	*13*
	C57BL/6J (F, 9 weeks)	↘ Seric and colonic 5-HT levels and colonic TPH1 expression, ↗SLC64A colonic expression	N/A	*22*
	C57BL/6J (M , 8W)	↘ Seric and colonic 5-HT levels and colonic TPH1 expression, ↗SLC64A colonic expression	N/A	*23*
	C57BL/6J mice (M, 12 weeks)	↘ colonic TPH1 and HTR1A expression ↗colonic and cecal HTR4 expression and ileal and cecal SLC64A expression	No differences	Our study
Antibiotic treated vs CV	C57BL/6N mice (M, 8-11weeks)	N/A	↗SERT gene expression in amygdala	*24*
	C57BL/6 mice (M 6-7 weeks)	↘ 5-HT protein levels, TPH1 gene expression, and number of 5-HT+ cells in colon	N/A	*25*

Table S2 : Summary of the findings in literature and our study for changes in the 5-HT metabolism in the gut or brain in GF, or antibiotic treated mice, compared to CV mice.

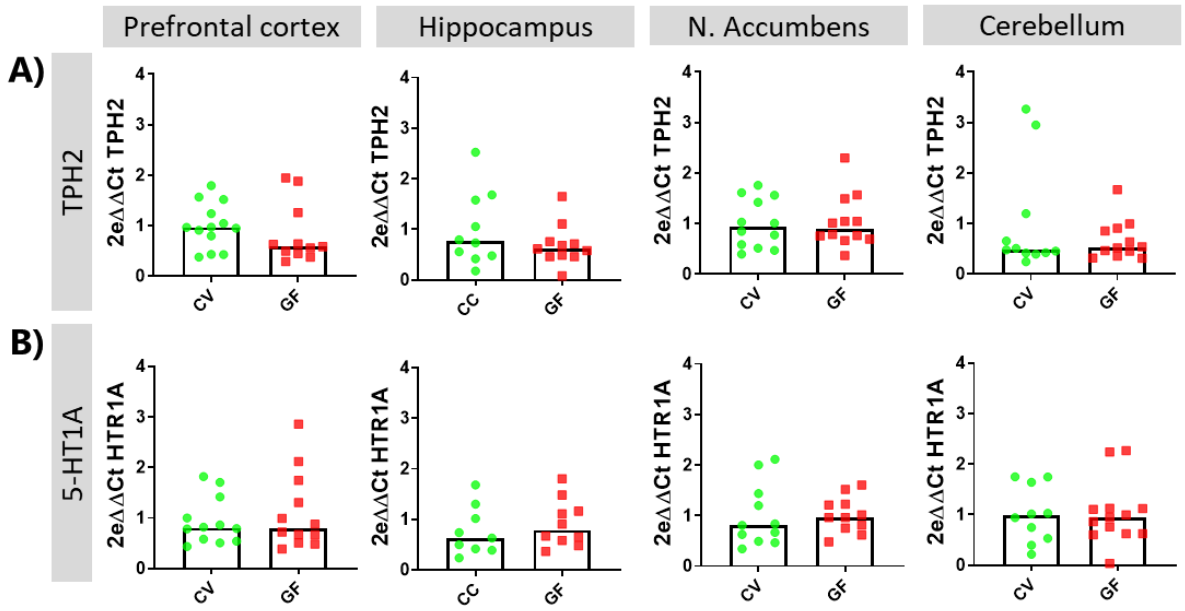


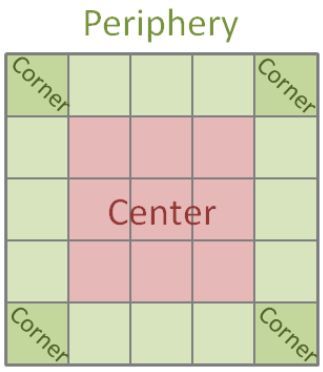
Figure S1: Relative gene expression of A) TPH2 and B) HTR1A in Prefrontal cortex, Hippocampus, Nucleus Accumbens (N. Accumbens) and Cerebellum of GF and CV mice. Expressed as 2eΔΔCT of target gene – housekeeping gene (ß-actin). A) Cerebellum: 1 outlier removed in GF group B) Hippocampus: 1 outlier removed in CV group.

13.2 Supplementary Materials and Methods:

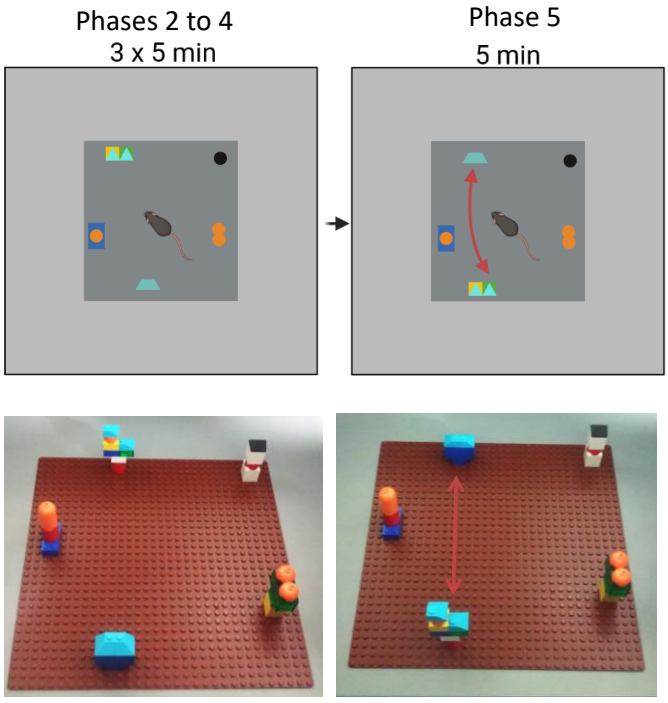
13.2.1 Creation of the CV line from recolonized GF mice:

As the GF colony bred in Anaxem was established years ago, there might have been a genetic drift leading to genetic differences in comparison to mice from a commercial supplier. Therefore, to obtain a CV strain genetically as close as possible to the GF mice, GF mice were colonized with fecal microbiota from sex- and age- matched SPF mice from the same strain (Charles River, Ecully, France), first by putting fecal pellets in their cages every 2 days for 3 weeks, then through one oral administration by having the mice suck on a pipette containing a suspension of 5 pellets in 1.5 mL of PBS 1X. Those progressively recolonized mice were bred to obtain successively F1 and F2 generations. The F2 generation was used in the study as the CV group.

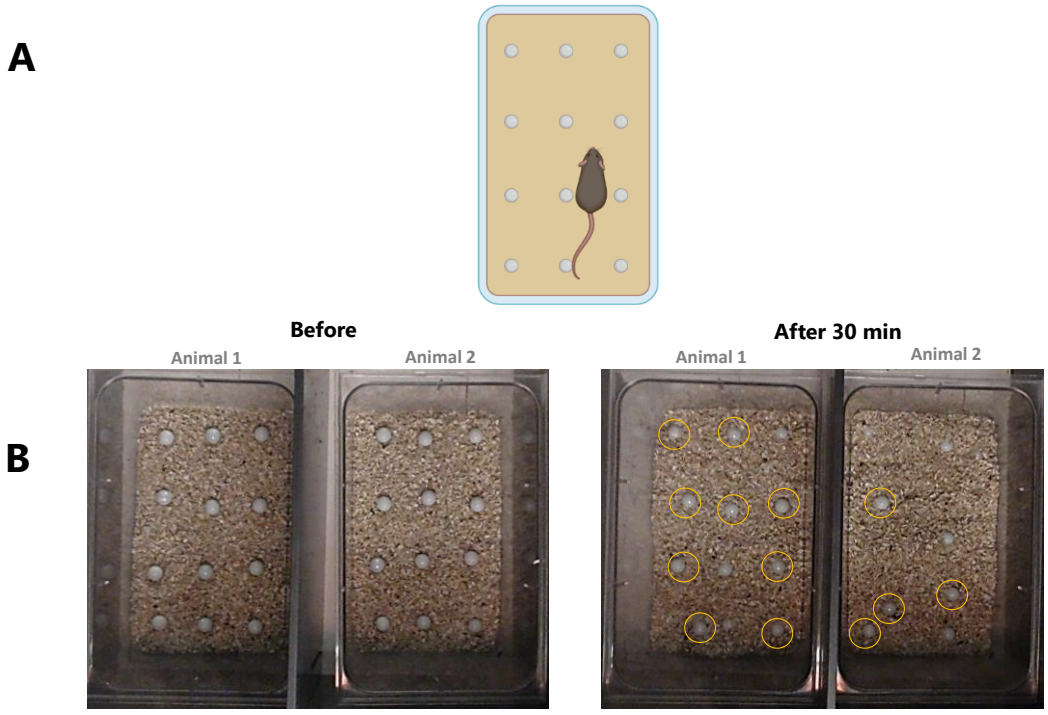
13.2.2 Supplementary schematic of behavioral tests:



Schematic representation of OF zones: The surface of the OF was divided into 25 equal squares and the 9 central squares constituted the central zone. The "corner" zones were made up of one square.



Schematic representation and picture of the objects in the 5-object recognition test.



Representation of the marble burying test A) Schematic of the marble burying test (created on Biorender.com) B) Picture of the test cages before and after 30 min of test. Circled in yellow are the marbles not buried after 30 min. Here, animal 1 buried 3 and animal 2 buried 8.

13.2.3 **Details of punches on brain slices for RNA extraction:**

Region and coordinates from the bregma	Number of slices	Number of punches by diameter			Total number of punches
		1 mm	1.2mm	2mm	
Prefrontal cortex *(from 3.08 to 2.1 mm)*	10	--	2	8	10
Accumbens *(from1.7 to 0.74 mm)*	10	--	10	--	10
Hippocampus *(from -1.46 to -2.54 mm)*	10	--	10	--	10
Cerebellum *(from -5.52 to -6.64 mm)*	11	11	--	11	22

Table 3 : Number of punches made in the different brain regions and localization according to *Paxinos and Franklin: "The Mouse Brain in Stereotaxic Coordinates" (2007)* [26] **.** Antero-posterior coordinates are given from the bregma (mm). Punchers of different diameters (1, 1.2 and 2 mm) were used to accurately delimitate each region on different slices.

13.2.4 **Table of primers used for qPCR:**

Intestine			Brain		
Marker	**Reference**	**Dye**	**Marker**	**Reference**	**Dye**
β-Actin	Mm02619580_g1	VIC	***β-Actin***	Mm02619580_g1	VIC
TPH1	Mm01202614_m1	FAM	***HTr1a***	Mm00434106_s1	FAM
Slc6a4	Mm00439391_m1	FAM	***TPH2***	Mm00557722_m1	FAM
HTr1a	Mm00434106_s1	FAM			
HTr4	Mm00434129_m1	FAM			

Table 4: Details of the primers used for qPCR. (TaqMan™ Gene Expression Assay (Applied Biosystems, Fisher Scientific)).

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