Alterations of brain endocannabinoidome signaling in germ-free mice

Claudia Manca[a,b,f], Melissa Shen[a,b,f], Besma Boubertakh[a,b,f], Cyril Martin[a,b,f], Nicolas Flamand[a,b,f], Cristoforo Silvestri*[a,b,f], Vincenzo Di Marzo*[a,b,c,d,e,f]

[a] Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec (IUCPQ), Québec, Canada;

[b] Département de médecine, Faculté de Médecine, Université Laval, Québec, Canada;

[c] Institut sur la nutrition et les aliments fonctionnels (INAF), Québec, Canada;

[d] École de nutrition, Faculté des sciences de l'agriculture et de l'alimentation (FSAA), Université Laval,Québec, Canada;

[e] Joint International Unit between the National Research Council (CNR) of Italy and Université Laval on Chemical and Biomolecular Research on the Microbiome and its Impact on Metabolic Health and Nutrition (UMI-MicroMeNu), Institute of Biomolecular Chemistry, CNR, Pozzuoli, Italy;

[f] Canada Research Excellence Chair on the Microbiome-Endocannabinoidome Axis in Metabolic Health (CERC-MEND).

*to whom correspondence should be addressed

vincenzo.dimarzo@criucpq.ulaval.ca

cristoforo.silvestri@criucpq.ulaval.ca

Abstract

We investigated the hypothesis that the endocannabinoidome (eCBome), an extension of the endocannabinoid (eCB) signaling system with important functions in the CNS, may play a role in the microbiota-gut-brain axis. Using LC-MS/MS and qPCR arrays we profiled the brain eCBome of juvenile (4 weeks) and adult (13 weeks) male and female germ-free (GF) mice, which are raised in sterile conditions and virtually devoid of microbiota, present neurophysiological deficits, and were found recently to exhibit a strongly altered gut eCBome in comparison to conventionally raised age/sex-matched controls. The causal effect of the gut microbiome on the eCBome was investigated through the re-introduction into adult male GF mice of a functional gut microbiota by fecal microbiota transfer (FMT). The concentrations of the eCB, 2-arachidonoylglycerol (2-AG), and its 2-monoacylglycerol congeners, were significantly reduced in the brain, but not in the hypothalamus, of both juvenile and adult male and adult female GF mice. FMT rendered these decreases non-statistically significant. The eCB, anandamide (AEA), and its congener *N*-acylethanolamines (NAEs), were instead increased in the brain of adult female GF mice. Saturated fatty acid-containing NAEs were decreased in adult male GF mouse hypothalamus in a manner not reversed by FMT. Only few changes were observed in the expression of eCBome enzymes and receptors. Our data open the possibility that altered eCBome signaling may underlie some of the brain dysfunctions typical of GF mice.

Key words: endocannabinoids, endocannabinoidome, microbiota, microbiome, gut-brain axis, fecal microbiota transfer

Running title: Gut microbiota affect the brain endocannabinoidome

1. Introduction

The gut microbiota is a critical regulator of host physiology [1]. Apart from extremely restrictive laboratory conditions, mammals have always coexisted with commensal microbes and, therefore, among the signals that the brain receives from the gut there have always been also those influenced by, or directly deriving from, the microorganisms living there [2]. The bidirectional communication between the gut microbiota and the brain, referred to as 'microbiota-gut-brain axis' [1] includes not only intricate neuronal pathways but also small molecule messaging systems such as microbiota-derived neuromodulatory metabolites (tryptophan precursors and metabolites, serotonin, GABA and catecholamines) and gut-derived cytokines, chemokines, neurotransmitters, neuropeptides and hormones. These molecules update the brain on the health status and possibly regulate behavior [1].

The gut microbiota is characteristically different during lifespan and it has been hypothesized that the periods of its major transformation occurring at both extremes of life may be likened to sensitive periods in the development or decline of other systems including, among others, the hypothalamic-pituitary-adrenal (HPA) axis and the hippocampal serotonergic system, thus potentially underlying the development of neural disorders [3–5]. Alterations in the microbiota can modulate neuronal activity and host behavior; however, the mechanisms remain poorly defined [6]. The use of mice lacking microbial colonization since birth (known as germ-free [GF] mice) represents a helpful tool in parsing the role of the microbiota in gut-brain signaling [7] and in the regulation of the behavior and neurophysiology of the host across lifespan [8]. Animal studies indicate that the absence or modification of the intestinal microbiota affects adult hippocampal neurogenesis [9], microglial maturation and myelination [10], blood-brain barrier integrity and permeability [11], as well as stress-related responses and fear learning [12–14]. The causal effects of microbiota on behavioral phenotypes or their disturbances [1] can be transferred or reversed, even between different species, by fecal microbiota transplant (FMT) [15–18].

We have recently shown that the most proximal site potentially affected by the lack of the gut microbiota, i.e. the small and large intestine, of juvenile and adult GF male mice exhibit strong modifications in the concentrations of the lipid mediators, and in the gene expression of receptors and metabolic enzymes, belonging to the endocannabinoidome (eCBome) [19]. This is a complex lipid signaling system deriving from the extension of the endocannabinoid (eCB) system to eCB-like mediators [20], which has been suggested to communicate with the gut microbiota in the context of metabolic disorders [21]. Further, we showed that FMT from adult donors to age-matched GF mice led to the partial or complete reversal of many of these changes after only one week, leading us to conclude that many of the observed modifications were directly due to the lack of microbiota.

These data prompted us to investigate the possible implication of eCBome signaling in the microbiota-gut-brain axis, which is suspected to involve several eCBome receptors expressed in the brain, such as the cannabinoid CB1 receptor, G-protein-coupled receptors 55 and 119 (GPR55 and GPR119), peroxisome

proliferator activated receptor-α (PPARα) and transient receptor potential vanilloid type-1 (TRPV1) channels [22]. Indeed, apart from its well-known function in metabolism, reproduction, growth and development [23], the eCBome acts throughout the nervous system to regulate neuronal synaptic communication, and affects central functions such as eating behavior, stress-related disorders, learning, memory and fear conditioning extinction [24,25]. The eCBome receptors and signals are also involved in the regulation of embryonic and adult neurogenesis [26,27] and particularly in the proliferation, differentiation, survival and migration of progenitor stem cells and their progeny in the adult brain [28]. Thus, we hypothesized that a dysfunctional eCBome in the brain of these mice could underlie part of the developmental alterations observed in GF mice. We, therefore, utilized the same GF and conventionally raised (CR) mice used in our previous study [19] to profile the brain eCBome, in both sexes and in both juvenile and adult mice. Additionally, in adult male mice, we further investigated the role of the gut microbiome in the regulation of the brain eCBome by analyzing the effect of FMT.

2. Materials and Methods

2.1. Animals and housing

Conventionally raised (CR) and germ-free (GF) C57BL/6NTac mice were purchased from Taconic (#B6 and #GF-B6, Taconic Bioscience, NY, USA) and maintained in the animal facility of the Institut Universitaire de Cardiologie et de Pneumologie de Québec (IUCPQ, QC, Canada). All animals were grouped 3-4 mice per cage under a 12h:12h light dark cycle with *ad libitum* access to NIH-31 Open Formula Autoclavable Diet (Zeigler, PA, USA) and water. GF mice were housed in axenic status, and fecal samples were weekly tested for microbes and parasites by the facility's staff to ensure that the GF unit was indeed sterile. Both GF and CR mice were acclimatized for at least one week prior to starting with the procedures. For each group of mice, the sacrifices started always at the same time, 12:30 pm.

2.2. Animal experiments and Fecal Microbial Transplant (FMT)

12 (6 male and 6 female) CR and GF mice at 4 and 13 wks of age with an average of weight of 15gr and 27gr for young and adult male mice and 13gr and 22gr for female mice, were intraperitoneally anesthetized with a cocktail of ketamine/xylazine/acepromazine which represent a balanced anesthetic combination with rapid induction time and long duration, at a dose of 50/10/1.7 mg/kg body weight and euthanized by cervical dislocation, CR and GF male mice first and female mice subsequently, following an intra-cardiac puncture.
The head of the mice was separated from the body for brain isolation and subsequent hypothalamus isolation. Other brain areas were not dissected because of the smaller size of the 4 wk mouse brain and the necessity of reducing the post-mortem delay, which is known to affect tissue eCB levels. With a pair of Iris scissors, the muscle behind the head, and the skin was removed. The scissors were inserted in the occipital hole, about 5mm deep, to cut the interparietal bone in the middle, right and left. Then, the Iris scissors were placed in the bregma and a light pressure was applied until blood came out. The sagittal suture skull was then opened, and the skull was removed. The brain was reversed, and the optic chiasma was disconnected, and with a small spatula, the brain was removed from the cavity. The hypothalamus was isolated using curved forceps and the brain was divided, using a scalpel, in the two hemispheres which were immediately snap frozen and stored at -80°C (the left) and in RNALater (#AM7021, Thermo Fisher Scientific, MA, USA) for RNA stabilization (the right). All the experimental protocols were validated and approved by the Université Laval animal ethics committee (CPAUL 2018010-1) and the study was not pre-registered.
For fecal microbiota transplant (FMT) experiments we only utilized 12wk-old male mice. GF mice were randomly divided into two groups at the age of 12wk; those gavaged with sterile PBS (SHAM; 5 mice) and those gavaged with fecal material (FMT; 6 mice). Material gavaged for FMT consisted of a cocktail of the intestinal contents and stools of a single and 4 CR donor mice, respectively. Briefly, the intestinal contents of the duodenum, jejunum, ileum, colon, and cecum were collected from one 12-week-old CR donor mouse and mixed with stool pellets from all the CR mice to be used as controls. The mixture was well homogenized, weighed, suspended 1:10 in sterile PBS, and centrifuged at 805 g for 10 min at room temperature. The supernatant was used to gavage the mice (200 µl of homogenate per mouse) [19]. The FMT mice were then

housed (3 per cage, like for CR mice) for one week in conventional conditions in cages contaminated with used litter coming from donor mouse cages. SHAM mice were submitted to a similar gavage with saline solution, but then kept in the germ-free facility for a week in the same conditions as GF mice. CR mice were euthanized the day of the gavages, while SHAM and FMT mice were sacrificed one week after the gavage; the tissues were collected from all animals randomly and as previously described (for an outline of the experimental protocol see Scheme 1). No statistical methods were employed to predetermine the sample size of the experiment and no exclusion criteria was applied.

2.3. Lipid extraction and HPLC-MS/MS for the analysis of eCBome mediators

We analyzed separately the hypothalamus and the whole brain minus the hypothalamus. Lipids were extracted from tissue samples according to the Bligh and Dyer method [29] with slight modifications. Tissues were processed and analyzed randomly and blindly. Briefly, the left hemisphere of each mouse was powdered in liquid nitrogen or an entire hypothalamus and about 10mg were homogenized in 1ml of a 1:1 Tris-HCl 50mM pH 7: methanol solution containing 0.1M acetic acid and 5ng of deuterated standards. The hypothalamus followed the same steps but were directly homogenized in the 1:1 Tris-HCl solution with a tissue grinder and not powered in liquid nitrogen. 1ml of chloroform was then added to each sample, which was then vortexed for 30 seconds and centrifuged at 3000×g for 5 minutes. The organic phase was collected and another 1 ml of chloroform was added to the inorganic one. This was repeated twice to ensure the maximum collection of the organic phase. The organic phases were pooled and evaporated under a stream of nitrogen and then suspended in 50µl of mobile phase containing 50% of solvent A (water + 1mM ammonium acetate + 0,05% acetic acid) and 50% of solvent B (acetonitrile/water 95/5 + 1mM ammonium acetate + 0.05% acetic acid). 40µl of each sample was finally injected onto an HPLC column (Kinetex C8, 150 × 2.1mm, 2.6µm, Phenomenex) and eluted at a flow rate of 400µl/min using a discontinuous gradient of solvent A and solvent B [30]. Quantification of eCBome-related mediators (suppl. Table 1), was carried out by HPLC system interfaced with the electrospray source of a Shimadzu 8050 triple quadrupole mass spectrometer and using multiple reaction monitoring in positive ion mode for the compounds and their deuterated homologs.

In the case of unsaturated monoacyl-glycerols, the data are presented as 2-monoacylglycerols (2-MAGs) but represent the combined signals from the 2- and 1(3)-isomers since the latter are most likely generated from the former via acyl migration from the *sn*-2 to the *sn*-1 or *sn*-3 position.

2.4. RNA isolation and Reverse Transcription

For each mouse, the right hemisphere or whole hypothalamus was ground in liquid nitrogen with a mortar and pestle, and RNA was extracted from about 10mg of the powder using the RNeasy Lipid Tissue Mini Kit (#74804, Qiagen, Hilden, Germany) following the manufacturer's instructions and eluted in 30µl of UltraPure Distilled Water (#10977035, Invitrogen, CA, USA). Tissues were processed randomly and blindly. The concentration and purity of RNA were determined by measuring the absorbance at 260nm and 280nm, and RNA integrity was assessed by an Agilent 2100 Bioanalyzer, using the Agilent RNA 6000 Nano Kit (#5067-1511, Agilent Technologies, CA, USA). One microgram of total RNA was reverse transcribed using a High-

Capacity cDNA Reverse Transcription Kit (#4368814, Applied Biosystems, CA, USA) in a reaction volume of 20µl.

2.5. QPCR-based TaqMan Open Array and Real-Time Fluorescence quantitative PCR (qPCR)

Sixty-five nanograms total of starting RNA were used to evaluate the expression of the 52 eCBome-related genes and 4 housekeeping genes (Suppl. Table 2) using a custom-designed qPCR-based TaqMan Open Array on a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific, CA, USA) following the manufacturer's instructions. Samples were analyzed randomly.

Ten nanograms of starting RNA were used to evaluate the expression of 11 neurogenesis and stress-related genes by QPCR using custom-designed primers (IDT, IA, USA) (Suppl. Table 3) and PowerUp SYBR Green QPCR master mix (#A25778, Applied Biosystem) according to the manufacturer's recommendations on a CFX384 Touch QPCR system (Bio-Rad).

The mRNA expression levels were calculated from duplicate reactions using the $2^{-\Delta\Delta Ct}$ method as calculated by CFX Maestro Software (Bio-Rad) and are represented as fold change with respect to baseline within each tissue for each age. *Hprt1* (Mm.PT.39a.22214828, IDT, IA, USA) and *Tbp* (Mm.PT.39a.22214839, IDT, IA, USA) were used as reference genes.

2.6. Statistical analysis

Data are presented as boxes (mean value) and whisker (higher and lower values) and as the mean±standard error of the mean (S.E.M), as specified in the individual Figures and Tables respectively.

The data were analyzed as follows: the differences between the CR and GF of 4 wks and 13 wks of age were assessed separately for each sex, and the brain and hypothalamus were analyzed independently. The 13 wk-old control results were assembled into a single control group. Differences between CR and GF 4 and 13 wk-old male and female mice were assessed using an unpaired Mann-Whitney T-test on ΔΔdCt and on pmol/mg tissue for gene expression levels and mediator levels respectively. In the 13 wk-old male mice where the FMT was performed, SHAM and FMT group statistical analyses were relative to the 13wk male GF group and the differences were determined by a One-Way ANOVA followed by Fisher's Least Significant Difference (LSD) test. For both the 4 and 13 wk-old mouse analysis (analyzed separately), the differences between experimental groups were considered statistically significant with $p \leq 0.05$. Data were analyzed using the GraphPad Prism 6.01 software (La Jolla, CA, USA).

3. Results

3.1. Endocannabinoid levels are altered in the brain and hypothalamus of GF mice

First, we investigated if the absence of the gut microbiota affects eCB levels within the whole brain and the hypothalamus of juvenile (4wk) and adult (13 wks) GF and CR male and female mice. In adult male mice, we also performed an FMT to conventionalize 12 wk-old GF mice and we reassessed eCB levels after one week to determine if introduction of a functional gut microbiota could reverse the observed changes. The FMT was successful, as shown in our previous study (see [19], Fig. 6), as the mice used in the present study were the same as those used in the previous one.

Concerning AEA in the whole brain, while a slight and non-statistically significant decrease was observed in juvenile GF male mice and no changes were present at 13wk of age before and after FMT, GF female mice showed a slight trend towards increased levels that became significant at 13wk of age (Fig. 1A). In the hypothalamus, only non-statistically significant trends were observed (Table 1).

As for the more abundant eCB, i.e. 2-AG, the levels were significantly decreased in the whole brain of young GF male mice and adult GF male as well as in adult female mice. The FMT partially, although not significantly, reversed the observed decrease at 13wk of age (Fig. 1B). In the hypothalamus, no changes were statistically significant, although adult female GF mice showed a strong trend towards increased levels as compared to CR mice ($P < 0.1$ for 2-AG) (Table 1).

Globally, these data suggest a reduced eCB tone in the brain of young and adult GF male mice, whereas no net effect was observed in 13wk old female mice considering the increase of AEA and decrease of 2-AG levels. Instead, eCB tone tended to increase in the hypothalamus with trending increases of both AEA and 2-AG in juvenile male and adult female GF mice. Overall, AEA levels appeared to be higher, and 2-AG levels instead lower, in the brain (but not hypothalamus) of female as compared to male mice, in partial agreement with previous studies [31].

3.2. Genes encoding neurogenetic and stress-related factors

As mentioned in the Introduction, the lack of intestinal microbiota can affect neurogenesis as well as stress-related responses. We therefore measured, in the whole brain and in the hypothalamus, a panel of 11 genes of which 9 were related to neurogenesis (*Bdnf, Arc, Sox9, Sox2, Nestin, NeuroD, Mcm2, Gfap, Blbp*) and 2 to stress (*Npas4, Egr1*), to determine if they were altered in 4 and 13wk GF male and female mice. Then, to assess the potential for a reinstated gut microbiome to reverse the observed changes, we conventionalized 12-week-old GF male mice through FMT and reassessed the gene expression levels after 1 week. As shown in Fig. 2 for the brain and Table 2 for the hypothalamus, only some neurogenesis markers were changed in at least one of the situations considered, while there was no change in stress-related genes (data not shown). We will briefly discuss hereafter only those genes presenting modifications (Fig. 2 and Table 2).

With regard to the member of the neurotrophin family, *Bdnf*, whilst in the brain there were no changes in its expression at 4 and 13 wk of age in GF mice of either sex, there was a trend towards decreased expression in the hypothalamus of adult GF male mice. Both the SHAM and FMT groups showed a significant decrease of *Bdnf* expression in the brain of male mice compared to 13wk old GF mice, while no changes were observed for the hypothalamus.

As for the gene encoding the neuroepithelial stem cell cytoskeletal protein Nestin and the transcription factor NeuroD, which are both involved (among others) in neuronal differentiation, we observed, in the brain, a statistically significant increase only for *Nestin* in young GF male mice, while in the hypothalamus, we did not observe any modification. *NeuroD*, however, tended to increase in both 4 and 13 wk-old male hypothalamus, where the reintroduction of the gut microbiota by FMT led to an even more marked increase, which was significant with respect to the adult GF group.

Concerning the proliferation marker minichromosome maintenance protein 2 (*Mcm2*), no modification at 4 wks of age was present in the brain or hypothalamus of GF male or female mice. However, *Mcm2* expression decreased significantly uniquely in the brains of 13 wk-old GF male mice, where the conventionalization with FMT did not cause any further modification.

Gfap, the gene encoding the glial fibrillary acidic protein which is a well-known marker for astrocytes, was instead increased, in the brain, in 13wk female GF mice, whereas in the hypothalamus of male mice, we could only observe a slight, and not statistically significant, decrease in adult mice, which was significantly reverted by the reintroduction of the gut microbiota with the FMT.

3.3. Endocannabinoid-like mediators

Together with the two eCBs, AEA and 2-AG, we also measured related *N*-acylethanolamines (NAEs) and 2-MAGs belonging to the eCBome, in order to understand how the absence of the gut microbiota and the subsequent FMT affect their concentrations. As observed above for AEA and 2-AG, the brains of female mice appeared to contain higher levels of NAEs and lower levels of 2-MAGs than those of males, whereas such difference was not observed in the hypothalamus (Fig. 3 and data not shown).

With regard to the brain levels of the NAEs (Fig. 3), the unsaturated fatty acid containing NAEs (*N*-oleoyl-ethanolamide, OEA; *N*-linoleoyl-ethanolamide, LEA) tended to decrease, but only *N*-docosahexaenoyl-ethanolamide (DHEA) presented a slight and significant decrease in young GF male mice. The situation was more interesting for female mice where *N*-palmitoyl-ethanolamide (PEA), OEA, LEA and DHEA levels, which were slightly but not significantly increased in juvenile mice, became significantly increased in 13wk old mice. The same trend was observed for *N*-stearoyl-ethanolamide (SEA), which however did not change in a statistically significant manner. In the hypothalamus (Table 3), the levels of saturated fatty acid-containing NAEs (PEA and SEA), were instead decreased significantly in adult GF male mice, however FMT did not revert this effect. No difference between CR and GF mice was observed in female mice hypothalamus.

Regarding the brain 2-MAGs (Fig. 4), whereas 2-palmitoyl-glycerol (2-PG) showed a significant decrease only in juvenile male mice, 2-oleoyl-glycerol (2-OG) and 2-linoleoyl-glycerol (2-LG) levels were also significantly decreased in 13wk old male mice. In female mice, only 2-LG presented a slight significant increase, and only in juvenile mice, while adult females showed trends for reductions in these 2-MAGs. Similarly, polyunsaturated 2-MAGs (2-docosahexaenoyl-glycerol; 2-DPG, 2-eicosapentaenoyl-glycerol; 2-EPG and 2-docospentaenoyl-glycerol; 2-DHG) were decreased in 4wk old male but not female mice, and were more strongly decreased in adult mouse brains regardless of sex (except for 2-DPG which only showed this trend in males). In adult male mice, FMT rendered the decreases observed for the 2-MAGs in GF mice no longer statistically significant vs. CR mice, and reverted it in a statistically significant manner only for 2-EPG. However, FMT mice did not exhibit statistically significant differences compared to either GF or SHAM mice.

As for the hypothalamus, we did not observe any statistically significant change in 2-MAG levels (data not shown).

N-arachidonoyl-glycine is a biologically active endogenous AEA–like molecule. While its levels in the hypothalamus and in the whole brain of female mice were below the detection limit, in the whole brain of GF male mice, we observed an interesting trend toward a decrease at 4 wk of age that became more pronounced (P=0.06) at 13wk of age in a manner that tended to be reverted by FMT, albeit not significantly (Fig. 5).

3.4. Genes encoding eCBome receptors and metabolic enzymes

To investigate if the changes observed in the levels of the eCBome mediators were accompanied by changes in the expression of eCBome receptors or due to changes in the expression in their anabolic and catabolic enzymes, we next measured a panel of eCBome-related genes [33] (Suppl. Table 2) in juvenile and adult male and female mice lacking the gut microbiota and after the conventionalization of adult GF male mice with FMT. Since much fewer significant changes were observed for eCBome mediators in the hypothalamus, where we could analyze only males and detected only very minor and non-statistically significant changes in the expression of these genes, we will only describe the results obtained in the whole brain minus the hypothalamus (Fig. 6).

Concerning the genes encoding eCBome-related receptors (Fig. 6A), in the whole brain of male mice, *Gpr119* was the only showing a decrease in 4wk old and an increase in the adult GF mice, whereas *Pparα* displayed a decrease in expression only in 13wk old mice. Instead, in female GF mice, *Cnr1* expression decreased in adult, while *Ppara* expression increased in juvenile, brains. In general, but not always, changes in *Pparα* expression seemed to follow changes in its endogenous ligands, PEA and OEA, which tended to be decreased in the brain of adult male GF mice and increased in juvenile female GF mice. On the contrary, the increase in the levels of PEA and OEA found in adult female mice was not accompanied by increased *Ppara* expression. In adult male mice, FMT reverted the increase of *Gpr119* expression in a manner selective over SHAM, as well as the reduction of *Ppara* expression, although unselectively over SHAM. Finally, FMT, but not SHAM, significantly increased *Cnr1* expression. It is worthwhile mentioning that two-week treatment of 6 week-old

male C57BL6 mice with an antibiotic cocktail was previously reported not to modify hippocampal *Cnr1* and *Trpv1* expression [32].

As for the eCBome anabolic and catabolic enzymes for eCBs and their congeners (Fig. 6B), significant results were observed for *Daglb* (a main anabolic enzyme for 2-AG), *Gde1*, *Gdpd1* and *Faah* (main anabolic and catabolic enzymes for AEA) in 13wk old male GF mice, the expression levels of which was significantly decreased. *Ptges*, encoding for prostaglandin E synthase, involved in the biosynthesis of prostamides and prostaglandin glycerol esters of type E, and *Abhd16a*, encoding for a proposed 2-AG degrading enzyme, were also down-regulated in adult male GF mice. Fewer changes were observed in female GF mouse brains, and mostly at 4wks of age, i.e. a decrease of a proposed 2-AG catabolic enzyme, *Mogat1*, and again of *Abhd16a*, while the degrading enzyme for monoacylglycerols, *Abdh6*, presented a slight increase. The only alteration seen in adult female GF mice was the increase of *Pla1a*, proposed to be involved in the biosynthesis of eCBs. FMT significantly reverted all alterations seen in adult male mice, although, with the only exception of *Gdpd1*, this reversal was also observed with SHAM. This suggests that the effect of FMT might be also due to the gavaging procedure, to which SHAM mice were also subjected, rather than to the re-introduction of the microbiota.

In summary, the observed changes in the expression of eCBome-enzymes did not correlate with the observed changes in eCBome mediator levels, and did not seem to be due to the lack of the gut microbiome.

4. Discussion

In this study, we aimed at answering the question of whether or not the brain eCBome, a complex system of lipid mediators, their receptors and anabolic/catabolic enzymes, which is involved in the regulation of several brain functions and various behavioral phenotypes and stress-related disorders, is altered in mice without a functional gut microbiome. Such putative alteration could implicate the eCBome in the 'microbiota-gut-brain axis', which is emerging as an important regulator of the same neurophysiological functions. Therefore, we utilized a model of juvenile and adult, male and female, GF mice, which lack microbiota, in comparison to their age- and sex-matched CR controls, and measured eCBome mediator levels and eCBome-related gene expression as well as the expression of a subset of neurogenetic and stress-related genes. We subsequently performed a FMT only in adult male GF mice and checked to see if any of the alterations observed in these mice was reverted by the introduction of a functional gut microbiota after one week. We report the occurrence of strong changes in eCB and eCB-like mediator levels in the brain of both male and female, juvenile and adult GF mice. Some of these changes, i.e. the decrease in 2-AG and 2-MAG levels observed in the whole brain minus the hypothalamus, were: 1) as strong in juvenile as in adult male GF mice, 2) not any longer statistically significant following FMT in adult male GF mice, and 3) found also in female GF mice, although only at the adult age. These findings overall suggest that brain eCBome signaling, particularly at the level of 2-AG and related mediators and in male mice, is altered in mice raised under sterile conditions. This alteration seems to

be due only in part to the lack of the gut microbiota in these mice. Other significant alterations, such as the increased AEA and NAE levels in the whole brain of female GF mice, or the decreased saturated NAE levels in the hypothalamus of male GF mice, were either observed again only in adult mice, or completely unaffected by FMT, respectively. Therefore, at least in the latter case, such alterations might be the result of adaptive mechanisms arising from, but not directly dependent on, raising the animals in a sterile environment.

Unsurprisingly, the extent, especially at the level of eCBome-related gene mRNA expression, of the alterations observed here in the brain (without the hypothalamus) was not as profound as that previously reported by us [19] in the large and, particularly, small intestine, which are the tissues likely to be most directly affected by the lack or presence of host-gut microbiota communication. Yet, of the two tissues examined here, i.e. the hypothalamus and the rest of the brain, the former, which is most likely to be directly exposed to changes in bloodstream chemicals or autonomic sympathetic nerve fiber activity from the periphery, was also the one that presented the least significant alterations in eCBome lipids in either sex (Figs. 1, 3, 4) and eCBome metabolic enzyme genes in males (data not shown). This may suggest that eCBome signaling in the rest of the male brain may be more influenced than the hypothalamus by the lack of the gut microbiome via mechanisms that are partly independent from the widely investigated chemical and neural afferent pathways deriving from the gut. Such mechanisms may, however, still include the response to typical commensal bacteria-derived metabolites, such as short and branched-chain fatty acids and secondary bile acids [34,35]. On the other hand, changes in hypothalamic NAE levels in either female or male GF mice were either not observed in juvenile mice or completely unaffected by FMT, respectively, suggesting, as mentioned above, that they are not the direct consequence of gut microbiome alterations.

As mentioned above, very few modifications were observed in the eCBome mediator anabolic/metabolic genes in the brain of mice lacking the gut microbiota. Such alterations, in adult male mice, were mostly reverted by the FMT, but also to the SHAM, procedure, thus casting doubts about the possibility that they are the result of the lack of the gut microbiome. This is in contrast with our previous findings in the small and large intestine, where several dramatic changes were observed in the expression of these genes that were reverted by FMT selectively over SHAM, and hence attributed directly to the lack of microbiota [19]. These results again are not surprising given the physical proximity of the gut microbiota to the gut compared with the brain. Regardless, the observed alterations in brain eCB and eCB-like mediator levels in GF vs. CR mice cannot be explained by changes in the mRNA expression of their metabolic enzymes, but might instead be related to alterations in enzyme activity or biosynthetic precursor levels. Indeed, this same conclusion was reached with regard to the alterations in the intestinal levels of eCB and eCB-like mediator levels despite the presence in the gut of much stronger alterations of metabolic gene expression [19].

A potentially important finding of this study is the observation of decreased 2-AG signaling in the brain of both male and female adult GF mice. In female mice, this decrease is unlikely to be compensated, in terms of signaling at cannabinoid CB1 receptors, by the observed increase in AEA levels, especially given the small, but statistically significant, decrease in the expression of the CB1-encoding gene, *Cnr1*. Decreased CB1

signaling in GF mice may account for several behavioral features of these animals. For example, adult GF mice (as well as antibiotic-treated mice) exhibit deficits in fear extinction, independently of the vagus nerve and alterations in the immune responses, suggesting that signals derived from the microbiota are necessary for optimal extinction of conditioned fear responses [6]. Based on the well-established role of eCB level elevation and subsequent CB1 activation in the mouse basolateral amygdala as one of the most important mechanisms underlying physiological extinction of aversive memories [24], our findings lead us to speculate that microbiota-derived signals may be necessary to reach the optimal 2-AG levels necessary to activate CB1 receptors in this context. In addition, our present finding, in male GF mice, of decreased levels of *N*-arachidonoyl-glycine, a mediator known to regulate optimal microglial function and migration [36,37], might also partly explain why antibiotic-treated mice exhibit dysfunctional microglia, in a manner that was suggested to affect the correct development of excitatory neuron dendritic spines in the medial prefrontal cortex, with subsequent impairment in extinction learning [6]. Our observation that FMT non-significantly attenuated the deficits in 2-AG and *N*-arachidonoyl-glycine levels is in agreement with the previous finding that such a procedure, in adult mice, is insufficient also at reverting the impaired extinction learning of GF mice, since, for this purpose, the reinstatement of a functional microbiota needed instead to be carried out in a precise developmental window in neonatal mice [6].

Indeed, decreased CB1 signaling could also underlie the neurodevelopmental deficits previously described for GF mice, for example in terms of defective neurogenesis, as well as their widely documented impaired stress response [3]. The gut microbiota can regulate the expression of neurotrophins to modify neural survival and differentiation and subsequently behavior [10]. Previous studies showed that depletion of the gut microbiota with antibiotics or being raised in sterile conditions both strongly impair adult neurogenesis in mice [38]. Here we found that GF mice presented no changes in two neural marker genes of the stress response, and only a few modest alterations in the mRNA expression of genes that are known to be involved in either the proliferation or differentiation of neural progenitor cells. Of these genes, *Mcm2* and *Gfap* were decreased and increased, respectively, in the brain of adult male or female GF mice, suggestive of decreased progenitor cell proliferation or increased differentiation into astrocytes. Given the pro-proliferative and neurogenic effects of CB1 and CB2 receptors on progenitor neural cells [39,40], these changes seem to correlate in part with the aforementioned reduction in 2-AG levels/CB1 expression observed in adult GF mice. By contrast, the increase in *Nestin* and *Gfap* expression in juvenile GF male mice would rather suggest increased progenitor cell proliferation/differentiation, in disagreement with the previous literature mentioned above, and also with the reduced 2-AG levels observed in these mice.

In fact, the observed changes in the genes involved in progenitor cell proliferation and differentiation might also correlate with alterations in other eCBome-related mediators or genes, also reported here. In the male hypothalamus, the trend decreases in *Bdnf, Nestin* and *Gfap* in GF mice seem to follow the decreases in PEA and SEA, for whose target, PPARα, there is evidence for a stimulatory role of proliferation of neuron and astrocyte precursors in zebrafish [41]. Increased levels of the neurotrophic GPR110 agonist, DHEA, also

known as synaptamide [42], and of the anti-inflammatory NAEs, PEA, DHEA and OEA, in adult female GF mice, instead could represent a negative feedback mechanism aimed at counterbalancing the increased astrogliogenesis, which is possibly evidenced by the increase in *Gfap*, an effect that, to the best of our knowledge, has not been previously reported in these animals. Given the paucity of previous data on the function of non-eCB 2-MAGs, such as 2-OG, 2-LG, 2-EPG and 2-DHG, it is not possible to make any strong speculation regarding the significance of the decreased levels of these compounds in both juvenile and adult male GF mice. The only known targets for these compounds are: 1) GPR119 (in the case of 2-OG, 2-LG and also of LEA, another NAE whose levels are increased significantly in adult female GF mice), for which we did find decreased or increased mRNA expression in either juvenile or adult male mice, respectively (the effect in adult mice being reverted by FMT selectively over SHAM), but no function in neurogenesis is known; and 2) TRPV1 - activated by all saturated and polyunsaturated 2-MAGs [43] - which instead was shown to inhibit neural progenitor cell proliferation [44] and may thus be involved in the increased expression of *Nestin* and *Gfap* in 4 week GF mice. At any rate, the role of 2-EPG in the context of the microbiota-gut-brain axis will deserve further investigation since its decrease in adult male GF mice was fully reverted by FMT.

In conclusion, we have presented here data suggesting that GF mice, a widely used model in studies on the neurobiological importance of the gut microbiome, present a strongly altered brain eCBome, a complex lipid signaling system involved in several physiopathological aspects of the CNS. Some of the observed alterations, namely the reduction in brain 2-MAG and *N*-arachidonoylglycine levels in adult male GF mice, appeared to be slightly attenuated by FMT, indicating that they might be directly due, at least to some extent, to the lack of the gut microbiome. Other alterations, such as those concerned with AEA and NAEs, appeared to be sex- and/or age-dependent. Age-dependent changes might provide useful insight into the role of the emerging gut microbiome-eCBome axis in brain development. As to the sex-dependent changes, future FMT studies will need to be performed also with adult female GF mice in order to understand the direct link of these changes with the gut microbiota, which is known to undergo to sex-dependent regulation [45–47]. Another limitation of this study was to have measured eCBome mediators and gene expression in the whole brain and not in distinct brain areas involved in specific functions (with the exception of the hypothalamus). As a consequence, the several potential physiopathological implications of the observed alterations, and particularly the understanding of whether or not they participate in some of the neurodevelopmental and behavioral features of GF mice, could only be speculated upon here, and will require *ad hoc* studies with specific brain regions, animal models and neurological and behavioral assessments. Despite these limitations, and its mostly descriptive nature, the present report suggests that changes in the brain eCBome may play a role in the microbiota-gut-brain axis, and need to be taken into account in studies using GF mice.

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References

[1] J.F. Cryan, K.J. O'Riordan, C.S.M. Cowan, K.V. Sandhu, T.F.S. Bastiaanssen, M. Boehme, M.G. Codagnone, S. Cussotto, C. Fulling, A.V. Golubeva, K.E. Guzzetta, M. Jaggar, C.M. Long-Smith, J.M. Lyte, J.A. Martin, A. Molinero-Perez, G. Moloney, E. Morelli, E. Morillas, R. O'Connor, J.S. Cruz-Pereira, V.L. Peterson, K. Rea, N.L. Ritz, E. Sherwin, S. Spichak, E.M. Teichman, M. van de Wouw, A.P. Ventura-Silva, S.E. Wallace-Fitzsimons, N. Hyland, G. Clarke, T.G. Dinan, The Microbiota-Gut-Brain Axis, Physiological Reviews. 99 (2019) 1877–2013. https://doi.org/10.1152/physrev.00018.2018.

[2] R.M. Stilling, S.R. Bordenstein, T.G. Dinan, J.F. Cryan, Friends with social benefits: host-microbe interactions as a driver of brain evolution and development?, Frontiers in Cellular and Infection Microbiology. 4 (2014). https://doi.org/10.3389/fcimb.2014.00147.

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

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

[5] S. Kim, H. Kim, Y.S. Yim, S. Ha, K. Atarashi, T.G. Tan, R.S. Longman, K. Honda, D.R. Littman, G.B. Choi, J.R. Huh, Maternal gut bacteria promote neurodevelopmental abnormalities in mouse offspring, Nature. 549 (2017) 528–532. https://doi.org/10.1038/nature23910.

[6] C. Chu, M.H. Murdock, D. Jing, T.H. Won, H. Chung, A.M. Kressel, T. Tsaava, M.E. Addorisio, G.G. Putzel, L. Zhou, N.J. Bessman, R. Yang, S. Moriyama, C.N. Parkhurst, A. Li, H.C. Meyer, F. Teng, S.S. Chavan, K.J. Tracey, A. Regev, F.C. Schroeder, F.S. Lee, C. Liston, D. Artis, The microbiota regulate neuronal function and fear extinction learning, Nature. 574 (2019) 543–548. https://doi.org/10.1038/s41586-019-1644-y.

[7] P. Luczynski, K.-A. McVey Neufeld, C.S. Oriach, G. Clarke, T.G. Dinan, J.F. Cryan, Growing up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior, International Journal of Neuropsychopharmacology. 19 (2016) pyw020. https://doi.org/10.1093/ijnp/pyw020.

[8] B.D. Weger, C. Gobet, J. Yeung, E. Martin, S. Jimenez, B. Betrisey, F. Foata, B. Berger, A. Balvay, A. Foussier, A. Charpagne, B. Boizet-Bonhoure, C.J. Chou, F. Naef, F. Gachon, The Mouse Microbiome Is Required for Sex-Specific Diurnal Rhythms of Gene Expression and Metabolism, Cell Metabolism. 29 (2019) 362-382.e8. https://doi.org/10.1016/j.cmet.2018.09.023.

[9] E.S. Ogbonnaya, G. Clarke, F. Shanahan, T.G. Dinan, J.F. Cryan, O.F. O'Leary, Adult Hippocampal Neurogenesis Is Regulated by the Microbiome, Biological Psychiatry. 78 (2015) e7–e9. https://doi.org/10.1016/j.biopsych.2014.12.023.

[10] G. Sharon, T.R. Sampson, D.H. Geschwind, S.K. Mazmanian, The Central Nervous System and the Gut Microbiome, Cell. 167 (2016) 915–932. https://doi.org/10.1016/j.cell.2016.10.027.

[11] V. Braniste, M. Al-Asmakh, C. Kowal, F. Anuar, A. Abbaspour, M. Toth, A. Korecka, N. Bakocevic, L.G. Ng, P. Kundu, B. Gulyas, C. Halldin, K. Hultenby, H. Nilsson, H. Hebert, B.T. Volpe, B. Diamond, S. Pettersson, The gut microbiota influences blood-brain barrier permeability in mice, Science Translational Medicine. 6 (2014) 263ra158-263ra158. https://doi.org/10.1126/scitranslmed.3009759.

[12] H.E. Vuong, J.M. Yano, T.C. Fung, E.Y. Hsiao, The Microbiome and Host Behavior, Annual Review of Neuroscience. 40 (2017) 21–49. https://doi.org/10.1146/annurev-neuro-072116-031347.
[13] A.E. Hoban, R.M. Stilling, G. Moloney, F. Shanahan, T.G. Dinan, G. Clarke, J.F. Cryan, The microbiome regulates amygdala-dependent fear recall, Molecular Psychiatry. 23 (2018) 1134–1144. https://doi.org/10.1038/mp.2017.100.
[14] J. Lu, S. Synowiec, L. Lu, Y. Yu, T. Bretherick, S. Takada, V. Yarnykh, J. Caplan, M. Caplan, E.C. Claud, A. Drobyshevsky, Microbiota influence the development of the brain and behaviors in C57BL/6J mice, PLOS ONE. 13 (2018) e0201829. https://doi.org/10.1371/journal.pone.0201829.
[15] P. Bercik, E. Denou, J. Collins, W. Jackson, J. Lu, J. Jury, Y. Deng, P. Blennerhassett, J. Macri, K.D. McCoy, E.F. Verdu, S.M. Collins, The Intestinal Microbiota Affect Central Levels of Brain-Derived Neurotropic Factor and Behavior in Mice, Gastroenterology. 141 (2011) 599-609.e3. https://doi.org/10.1053/j.gastro.2011.04.052.
[16] A.J. Bruce-Keller, J.M. Salbaum, M. Luo, E. Blanchard, C.M. Taylor, D.A. Welsh, H.-R. Berthoud, Obese-type Gut Microbiota Induce Neurobehavioral Changes in the Absence of Obesity, Biological Psychiatry. 77 (2015) 607–615. https://doi.org/10.1016/j.biopsych.2014.07.012.
[17] J.R. Kelly, Y. Borre, C. O' Brien, E. Patterson, S. El Aidy, J. Deane, P.J. Kennedy, S. Beers, K. Scott, G. Moloney, A.E. Hoban, L. Scott, P. Fitzgerald, P. Ross, C. Stanton, G. Clarke, J.F. Cryan, T.G. Dinan, Transferring the blues: Depression-associated gut microbiota induces neurobehavioural changes in the rat, Journal of Psychiatric Research. 82 (2016) 109–118. https://doi.org/10.1016/j.jpsychires.2016.07.019.
[18] P. Zheng, B. Zeng, C. Zhou, M. Liu, Z. Fang, X. Xu, L. Zeng, J. Chen, S. Fan, X. Du, X. Zhang, D. Yang, Y. Yang, H. Meng, W. Li, N.D. Melgiri, J. Licinio, H. Wei, P. Xie, Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism, Molecular Psychiatry. 21 (2016) 786–796. https://doi.org/10.1038/mp.2016.44.
[19] C. Manca, B. Boubertakh, N. Leblanc, T. Deschênes, S. Lacroix, C. Martin, A. Houde, A. Veilleux, N. Flamand, G.G. Muccioli, F. Raymond, P.D. Cani, V. Di Marzo, C. Silvestri, Germ-free mice exhibit profound gut microbiota-dependent alterations of intestinal endocannabinoidome signaling, Journal of Lipid Research. 61 (2020) 70–85. https://doi.org/10.1194/jlr.RA119000424.
[20] V. Di Marzo, J. Wang, The Endocannabinoidome The World of Endocannabinoids and Related Mediators, (2014).
[21] P.D. Cani, H. Plovier, M. Van Hul, L. Geurts, N.M. Delzenne, C. Druart, A. Everard, Endocannabinoids — at the crossroads between the gut microbiota and host metabolism, Nature Reviews Endocrinology. 12 (2016) 133–143. https://doi.org/10.1038/nrendo.2015.211.
[22] N. Forte, A.C. Fernández-Rilo, L. Palomba, V. Di Marzo, L. Cristino, Obesity Affects the Microbiota–Gut–Brain Axis and the Regulation Thereof by Endocannabinoids and Related Mediators, International Journal of Molecular Sciences. 21 (2020) 1554. https://doi.org/10.3390/ijms21051554.
[23] S.D. Skaper, V. Di Marzo, Endocannabinoids in nervous system health and disease: the big picture in a nutshell, Philosophical Transactions of the Royal Society B: Biological Sciences. 367 (2012) 3193–3200. https://doi.org/10.1098/rstb.2012.0313.
[24] G. Marsicano, C.T. Wotjak, S.C. Azad, T. Bisogno, G. Rammes, M.G. Cascio, H. Hermann, J. Tang, C. Hofmann, W. Zieglgänsberger, V. Di Marzo, B. Lutz, The endogenous cannabinoid system controls extinction of aversive memories, Nature. 418 (2002) 530–534. https://doi.org/10.1038/nature00839.
[25] K. Kamprath, H. Romo-Parra, M. Häring, S. Gaburro, M. Doengi, B. Lutz, H.-C. Pape, Short-Term Adaptation of Conditioned Fear Responses Through Endocannabinoid Signaling in the

Central Amygdala, Neuropsychopharmacology. 36 (2011) 652–663. https://doi.org/10.1038/npp.2010.196.
[26] R.W. de Oliveira, C.L. Oliveira, F.S. Guimarães, A.C. Campos, Cannabinoid signalling in embryonic and adult neurogenesis: possible implications for psychiatric and neurological disorders, Acta Neuropsychiatrica. 31 (2019) 1–16. https://doi.org/10.1017/neu.2018.11.
[27] J.A. Prenderville, Á.M. Kelly, E.J. Downer, The role of cannabinoids in adult neurogenesis: Cannabinoids and neurogenesis, British Journal of Pharmacology. 172 (2015) 3950–3963. https://doi.org/10.1111/bph.13186.
[28] S. Oddi, L. Scipioni, M. Maccarrone, Endocannabinoid system and adult neurogenesis: a focused review, Current Opinion in Pharmacology. 50 (2020) 25–32. https://doi.org/10.1016/j.coph.2019.11.002.
[29] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917. https://doi.org/10.1139/o59-099.
[30] A. Everard, H. Plovier, M. Rastelli, M.V. Hul, A. de W. d'Oplinter, L. Geurts, C. Druart, S. Robine, N.M. Delzenne, G.G. Muccioli, W.M. de Vos, S. Luquet, N. Flamand, V.D. Marzo, P.D. Cani, Intestinal epithelial N -acylphosphatidylethanolamine phospholipase D links dietary fat to metabolic adaptations in obesity and steatosis, Nature Communications. 10 (2019) 457. https://doi.org/10.1038/s41467-018-08051-7.
[31] H.B. Bradshaw, N. Rimmerman, J.F. Krey, J.M. Walker, Sex and hormonal cycle differences in rat brain levels of pain-related cannabimimetic lipid mediators, American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 291 (2006) R349–R358. https://doi.org/10.1152/ajpregu.00933.2005.
[32] F. Guida, F. Turco, M. Iannotta, D. De Gregorio, I. Palumbo, G. Sarnelli, A. Furiano, F. Napolitano, S. Boccella, L. Luongo, M. Mazzitelli, A. Usiello, F. De Filippis, F.A. Iannotti, F. Piscitelli, D. Ercolini, V. de Novellis, V. Di Marzo, R. Cuomo, S. Maione, Antibiotic-induced microbiota perturbation causes gut endocannabinoidome changes, hippocampal neuroglial reorganization and depression in mice, Brain Behav. Immun. 67 (2018) 230–245. https://doi.org/10.1016/j.bbi.2017.09.001.
[33] V. Di Marzo, C. Silvestri, Lifestyle and Metabolic Syndrome: Contribution of the Endocannabinoidome, Nutrients. 11 (2019) 1956. https://doi.org/10.3390/nu11081956.
[34] G. Caspani, J. Swann, Small talk: microbial metabolites involved in the signaling from microbiota to brain, Current Opinion in Pharmacology. 48 (2019) 99–106. https://doi.org/10.1016/j.coph.2019.08.001.
[35] J.L. McCarville, G.Y. Chen, V.D. Cuevas, K. Troha, J.S. Ayres, Microbiota Metabolites in Health and Disease, Annual Review of Immunology. 38 (2020) 147–170. https://doi.org/10.1146/annurev-immunol-071219-125715.
[36] D. McHugh, S.S. Hu, N. Rimmerman, A. Juknat, Z. Vogel, J.M. Walker, H.B. Bradshaw, N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor, BMC Neuroscience. 11 (2010) 44. https://doi.org/10.1186/1471-2202-11-44.
[37] D. McHugh, D. Roskowski, S. Xie, H.B. Bradshaw, Δ9-THC and N-arachidonoyl glycine regulate BV-2 microglial morphology and cytokine release plasticity: implications for signaling at GPR18, Frontiers in Pharmacology. 4 (2014). https://doi.org/10.3389/fphar.2013.00162.
[38] N. Sawada, T. Kotani, T. Konno, J. Setiawan, Y. Nishigaito, Y. Saito, Y. Murata, K. Nibu, T. Matozaki, Regulation by commensal bacteria of neurogenesis in the subventricular zone of adult mouse brain, Biochemical and Biophysical Research Communications. 498 (2018) 824–829. https://doi.org/10.1016/j.bbrc.2018.03.064.
[39] J. Díaz-Alonso, M. Guzmán, I. Galve-Roperh, Endocannabinoids via CB_1 receptors act as neurogenic niche cues during cortical development, Philosophical Transactions of the Royal

Society B: Biological Sciences. 367 (2012) 3229–3241. https://doi.org/10.1098/rstb.2011.0385.
[40] R. Rodrigues, D. Lourenço, S. Paulo, J. Mateus, M. Ferreira, F. Mouro, J. Moreira, F. Ribeiro, A. Sebastião, S. Xapelli, Cannabinoid Actions on Neural Stem Cells: Implications for Pathophysiology, Molecules. 24 (2019) 1350. https://doi.org/10.3390/molecules24071350.
[41] Y.-C. Hsieh, M.-C. Chiang, Y.-C. Huang, T.-H. Yeh, H.-Y. Shih, H.-F. Liu, H.-Y. Chen, C.-P. Wang, Y.-C. Cheng, Pparα deficiency inhibits the proliferation of neuronal and glial precursors in the zebrafish central nervous system: Pparα Regulates Neural Development, Developmental Dynamics. 247 (2018) 1264–1275. https://doi.org/10.1002/dvdy.24683.
[42] H.-Y. Kim, A.A. Spector, Synaptamide, endocannabinoid-like derivative of docosahexaenoic acid with cannabinoid-independent function, Prostaglandins, Leukotrienes and Essential Fatty Acids. 88 (2013) 121–125. https://doi.org/10.1016/j.plefa.2012.08.002.
[43] P.M. Zygmunt, A. Ermund, P. Movahed, D.A. Andersson, C. Simonsen, B.A.G. Jönsson, A. Blomgren, B. Birnir, S. Bevan, A. Eschalier, C. Mallet, A. Gomis, E.D. Högestätt, Monoacylglycerols Activate TRPV1 – A Link between Phospholipase C and TRPV1, PLoS ONE. 8 (2013) e81618. https://doi.org/10.1371/journal.pone.0081618.
[44] K. Stock, A. Garthe, F. de Almeida Sassi, R. Glass, S.A. Wolf, H. Kettenmann, The Capsaicin Receptor TRPV1 as a Novel Modulator of Neural Precursor Cell Proliferation: TRPV1 Modulates Neurogenesis, STEM CELLS. 32 (2014) 3183–3195. https://doi.org/10.1002/stem.1805.
[45] M.-C. Audet, Stress-induced disturbances along the gut microbiota-immune-brain axis and implications for mental health: Does sex matter?, Frontiers in Neuroendocrinology. 54 (2019) 100772. https://doi.org/10.1016/j.yfrne.2019.100772.
[46] M. Jaggar, K. Rea, S. Spichak, T.G. Dinan, J.F. Cryan, You've got male: Sex and the microbiota-gut-brain axis across the lifespan, Frontiers in Neuroendocrinology. 56 (2020) 100815. https://doi.org/10.1016/j.yfrne.2019.100815.
[47] E. Jašarević, K.E. Morrison, T.L. Bale, Sex differences in the gut microbiome–brain axis across the lifespan, Philosophical Transactions of the Royal Society B: Biological Sciences. 371 (2016) 20150122. https://doi.org/10.1098/rstb.2015.0122.

Legends to Figures and Tables

Scheme 1: Experimental protocols. A: twelve (6 male and 6 female) CR and GF mice were euthanized at 4 and 13 weeks of age. B: For FMT, 12-week-old GF mice were gavaged with either intestinal contents and stools coming from one and four donor mice, respectively (FMT group; n = 6), or sterile PBS (SHAM group; n = 5). For both experiments, brain and hypothalamus were isolated.

Figure 1 and Table 1: Concentrations in the hypothalamus (Table 1) and rest of the brain (Fig. 1) in 4 and 13wk old conventionally raised (CR) or germ-free (GF) male and female mice and in 13wk old male GF mice gavaged with sterile PBS (SHAM) or fecal microbiota (FMT). Levels of anandamide (AEA) (Fig. 1A) and 2-arachidonoyl-glycerol (2-AG) (the three mono-arachidonoyl-glycerol stereoisomers added together because presumably coming from the isomerization of 2-AG) (Fig. 1B) are expressed as pmol/mg of tissue. The statistical analysis compared 4 and 13wk old GF male and female mice with their age and sex-matched controls, while for brain SHAM and FMT the statistical analysis is relative to the 13wk male GF group within each tissue. Values are presented as boxes (mean value) and whisker (higher and lower values) in the Figure and as means ± S.E.M in the Table of n=5-10 for the brain and n=3 for the hypothalamus. *, $P \leq 0.05$; ***, $P \leq 0.005$.

Figure 2 and Table 2: Relative mRNA expression levels of genes encoding neurogenetic factors in the hypothalamus (Table 2) and rest of the brain (Fig. 2) in 4 and 13wk old conventionally raised (CR) or germ-free (GF) male and female mice and in 13wk old male GF mice gavaged with sterile PBS (SHAM) or fecal microbiota (FMT). The statistical analysis compared 4 and 13wk old GF male and female mice with their age and sex-matched controls, while for brain SHAM and FMT the statistical analysis is relative to the 13wk male GF group within each tissue. Values are presented as boxes (mean value) and whisker (higher and lower values) in the Figure and as means ± S.E.M in the Table of n=5-10 mice for brain and n=3-7 mice for hypothalamus. *, $P \leq 0.05$; **, $P \leq 0.01$

Figure 3 and Table 3: Concentrations in the hypothalamus (Table 3) and rest of the brain (Fig. 3) of endocannabinoid-like mediators in 4 and 13 wk-old conventionally raised (CR) or germ-free (GF) male and female mice and in 13 wk-old male GF mice gavaged with sterile PBS (SHAM) or fecal microbiota (FMT). Levels of eCB-like mediators are expressed as pmol/mg of tissue. The statistical analysis compared 4 and 13 wk-old GF male and female mice with their age and sex-matched controls, while for SHAM and FMT the statistical analysis is relative to the 13 wk-old male GF mouse group within each tissue. Values are presented as boxes (mean value) and whisker (higher and lower values) in the Figure and as means ± S.E.M in the Table of n=5-10 for the brain and n=3 for the hypothalamus. *, $P \leq 0.05$; **, $P \leq 0.01$.

PEA, *N*-palmitoyl-ethanolamide; SEA, *N*-stearoyl-ethanolamide; OEA, *N*-oleoyl-ethanolamide; LEA, *N*-linoleoyl-ethanolamide; DHEA, *N*-docosahexaenoyl-ethanolamide.

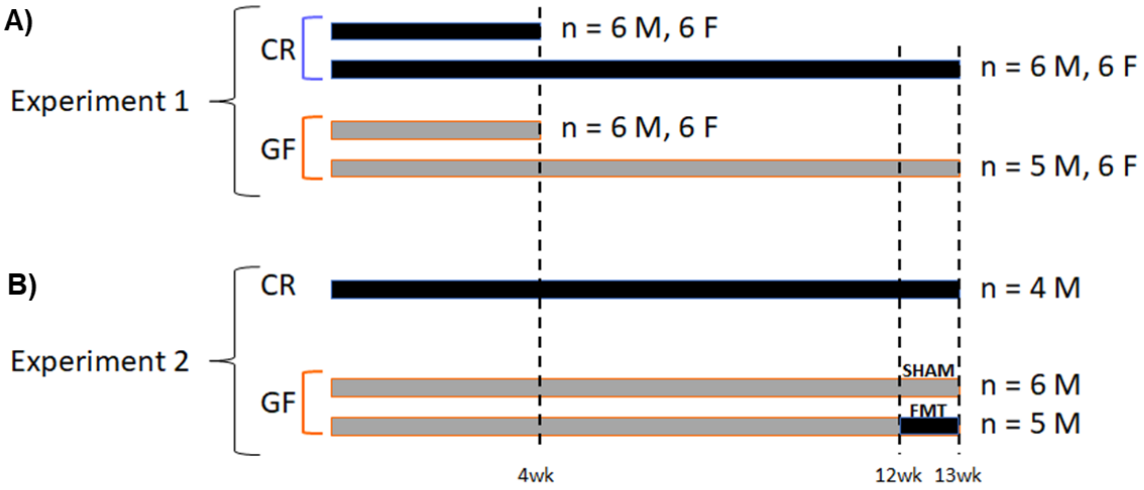
Figure 4: Concentrations in whole brain minus the hypothalamus of endocannabinoid-like mediators in 4 and 13 wk-old conventionally raised (CR) or germ-free (GF) male and female mice and in 13 wk-old male GF

mice gavaged with sterile PBS (SHAM) or fecal microbiota (FMT). Levels of eCB-like mediators are expressed as pmol/mg of tissue. The statistical analysis compared 4 and 13 wk-old GF male and female mice with their age and sex-matched controls, while for SHAM and FMT the statistical analysis is relative to the 13 wk-old male GF mouse group within each tissue. Values are presented as boxes (mean value) and whisker (higher and lower values) of n=5-10. *, $P \leq 0.05$; **, $P \leq 0.01$.

2-PG, 2-palmitoyl-glycerol; 2-OG, 2-oleoyl-glycerol; 2-LG, 2-linoleoyl-glycerol; 2-EPG, the three stereoisomers of mono-eicosapentaenoyl-glycerol (added together because presumably coming from the isomerization of 2-eicosapentaenoyl-glycerol); 2-DPG, the three stereoisomers of mono-docosapentaenoyl-glycerol (added together because presumably coming from the isomerization of 2-docosapentaenoyl-glycerol); 2-DHG, the three stereoisomers of mono-docosahexaenoyl-glycerol (added together because presumably coming from the isomerization of 2-docosahexaenoyl-glycerol).

Figure 5: Concentrations of *N*-arachidonoyl-glycine expressed as pmol/mg of tissue in whole brain minus the hypothalamus in 4 and 13 wk-old male and female conventionally raised (CR) or germ-free (GF) male and in 13 wk-old GF mice gavaged with sterile PBS (SHAM) or fecal microbiota (FMT). The statistical analysis compared 4 and 13 wk-old GF male and female mice with their age and sex-matched controls, while for SHAM and FMT the statistical analysis is relative to the 13 wk-old male GF mouse group. Values are presented as boxes (mean value) and whisker (higher and lower values) of n, 5-10.

Figure 6: Relative mRNA expression levels of genes encoding eCBome-related receptors (A) and metabolic enzymes for 2-acyl-glycerols and *N*-acylethanolamines (B) in whole brain minus the hypothalamus in 4 and 13wk old conventionally raised (CR) or germ-free (GF) male and female mice and in 13wk old male GF mice gavaged with sterile PBS (SHAM) or fecal microbiota (FMT). The statistical analysis compared 4 and 13wk old GF male and female mice with their age and sex-matched controls, while for brain SHAM and FMT groups the statistical analysis is relative to the 13wk male GF group within each tissue. Values are presented as boxes (mean value) and whisker (higher and lower values) in the Figure and as means ± S.E.M in the Table of n=5-6 mice for brain and n=3-7 mice for hypothalamus. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$; ****, $P \leq 0.001$


A)
Experiment 1
CR
GF
n = 6 M, 6 F
n = 6 M, 6 F
n = 6 M, 6 F
n = 5 M, 6 F
B)
Experiment 2
CR
GF
n = 4 M
SHAM
FMT
n = 6 M
n = 5 M
4wk
12wk
13wk

Scheme 1

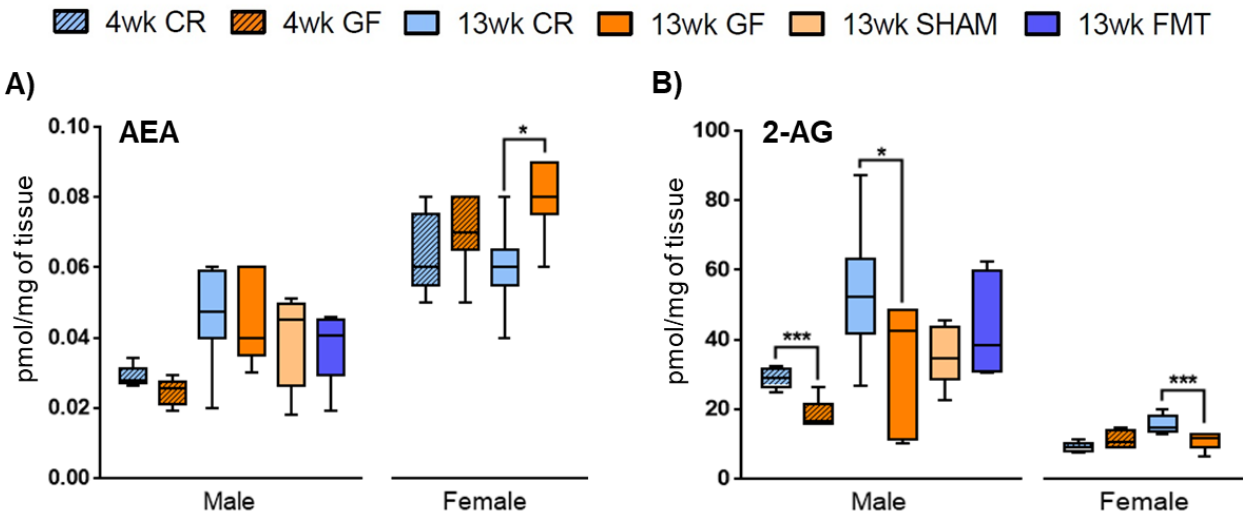


Fig. 1

	Male mice						Female mice			
Hypothalamus	4wk CR	4wk GF	13wk CR	13wk GF	13wk SHAM	13wk FMT	4wk CR	4wk GF	13wk CR	13wk GF
AEA	0.064 ± 0.004	0.089 ± 0.027	0.053 ± 0.006	0.059 ± 0.013	0.055 ± 0.013	0.079 ± 0.037	0.075 ± 0.023	0.040 ± 0.022	0.077 ± 0.018	0.133 ± 0.012
2-AG	13.138 ± 1.840	22.443 ± 11.295	16.553 ± 2.225	20.673 ± 5.256	17.964 ± 4.591	29.722 ± 2.999	11.080 ± 3.336	11.146 ± 3.387	20.632 ± 1.453	32.7 ± 4.551

Table 1

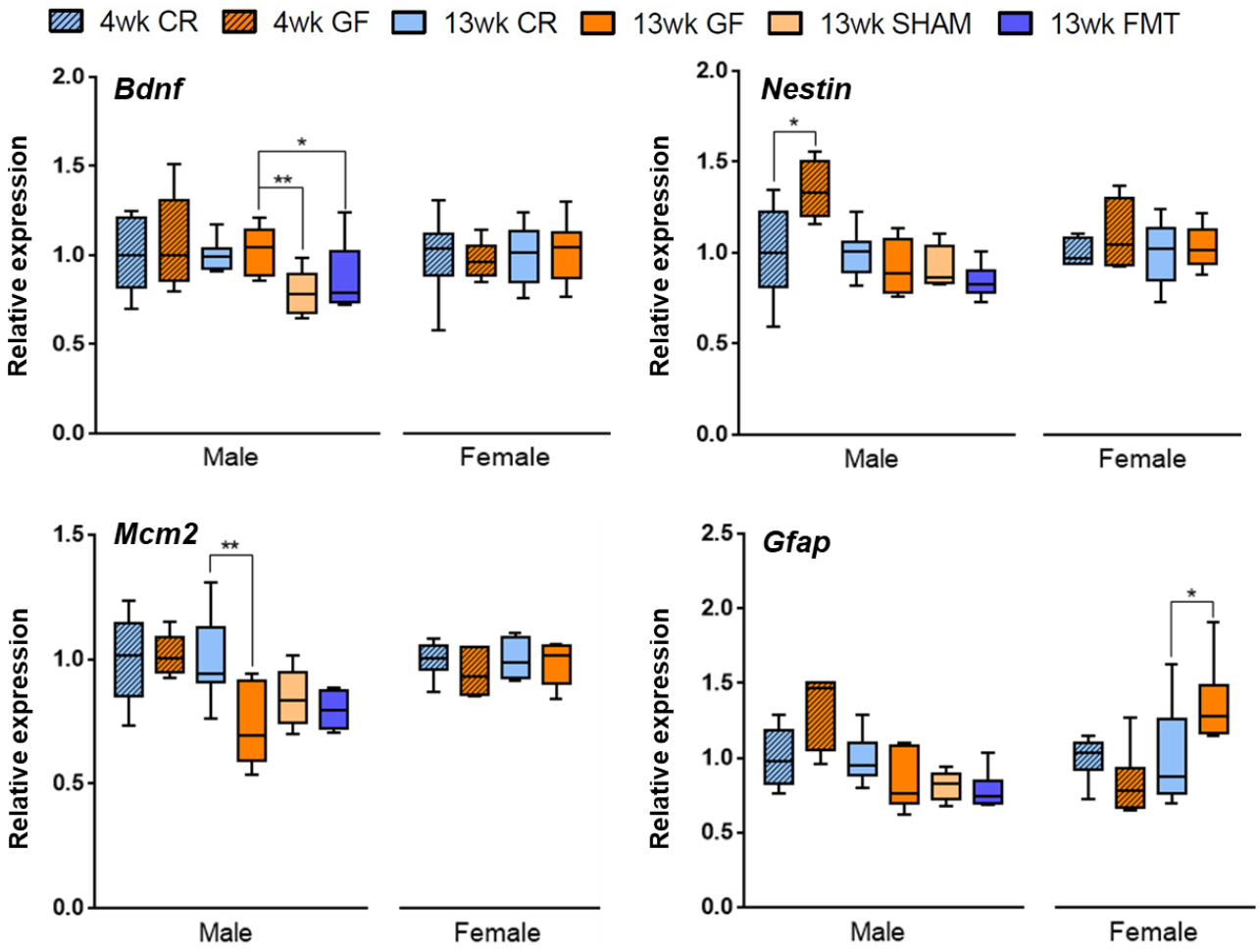


Fig. 2

	Male mice					
Hypothalamus	**4wk CR**	**4wk GF**	**13wk CR**	**13wk GF**	**13wh SHAM**	**13wk FMT**
Bdnf	1 ± 0.013	1.179 ± 0.143	1 ± 0.053	0.774 ± 0.009	0.767 ± 0.168	0.783 ± 0.030
Nestin	1 ± 0.018	0.999 ± 0.030	1 ± 0.113	0.790 ± 0.151	0.853 ± 0.166	0.690 ± 0.046
NeuroD	1 ± 0.117	1.843 ± 0.533	1 ± 0.083	1.464 ± 0.589	1.848 ± 0.095	*3.163 ± 0.590
Mcm2	1 ± 0.055	1.072 ± 0.030	1 ± 0.144	1.147 ± 0.210	1.988 ± 0.182	1.183 ± 0.107
Gfap	1 ± 0.046	0.934 ± 0.111	1 ± 0.098	0.680 ± 0.295	0.930 ± 0.013	*1.146 ± 0.012

Table 2

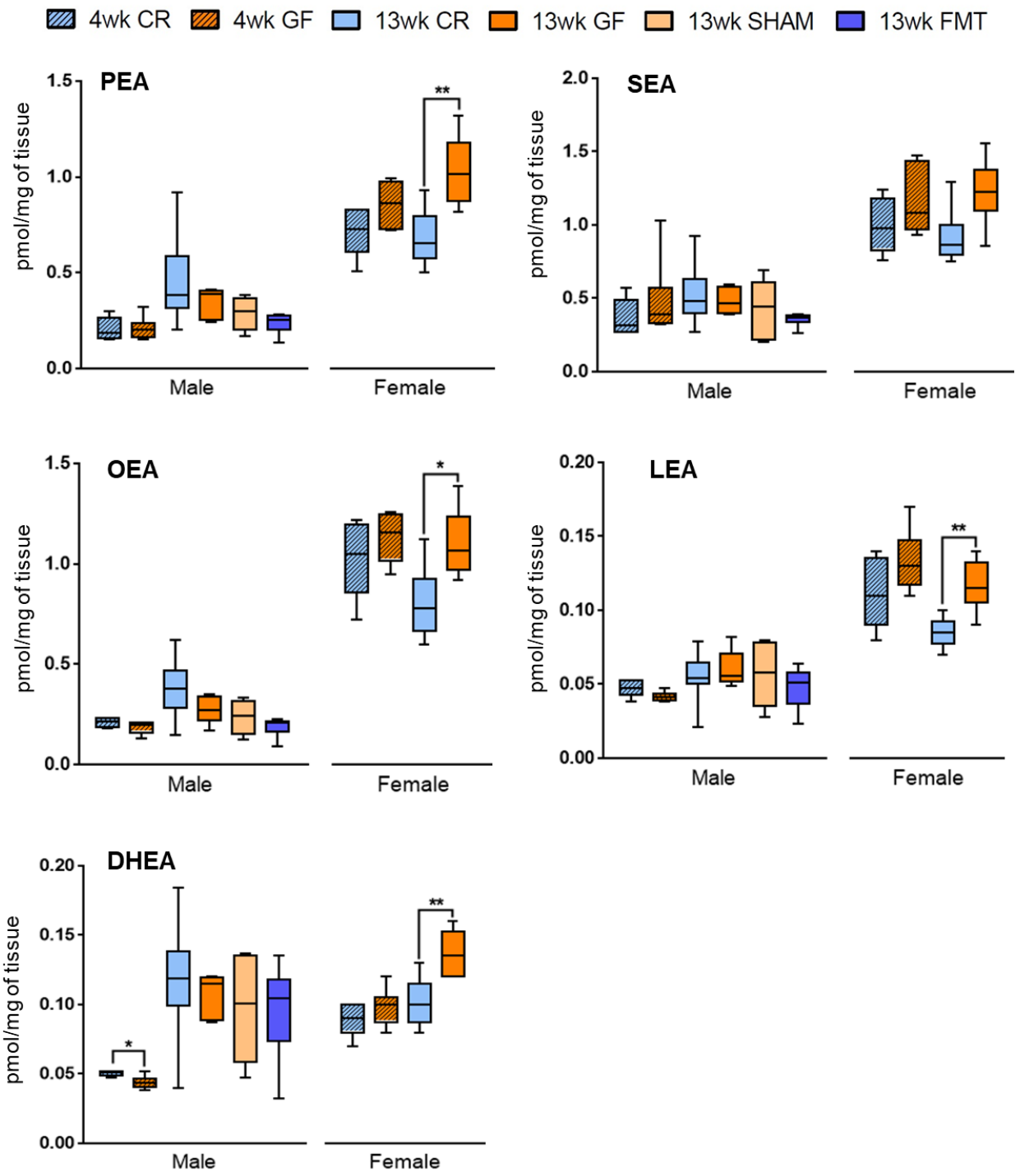


Fig. 3

	Male mice						Female mice			
Hypothalamus	4wk CR	4wk GF	13wk CR	13wk GF	13wk SHAM	13wk FMT	4wk CR	4wk GF	13wk CR	13wk GF
PEA	0.286 ± 0.003	0.394 ± 0.113	0.458 ± 0.037	**0.251 ± 0.023	0.270 ± 0.043	0.241 ± 0.056	0.371 ± 0.101	0.253 ± 0.122	0.411 ± 0.021	0.496 ± 0.019
SEA	0.478 ± 0.018	0.692 ± 0.184	1.050 ± 0.183	**0.424 ± 0.025	0.396 ± 0.057	0.439 ± 0.094	0.582 ± 0.1	0.416 ± 0.192	0.597 ± 0.045	0.592 ± 0.046
OEA	0.711 ± 0.235	1.227 ± 0.330	0.843 ± 0.145	0.778 ± 0.125	0.630 ± 0.114	0.645 ± 0.167	0.556 ± 0.138	0.797 ± 0.422	1.048 ± 0.221	1.172 ± 0.149
LEA	0.052 ± 0.002	0.082 ± 0.028	0.043 ± 0.004	0.037 ± 0.005	0.039 ± 0.007	0.026 ± 0.006	0.064 ± 0.02	0.035 ± 0.2	0.045 ± 0.006	0.067 ± 0.004
DHEA	0.048 ± 0.0009	0.059 ± 0.020	0.047 ± 0.005	0.046 ± 0.008	0.049 ± 0.008	0.040 ± 0.005	0.056 ± 0.016	0.029 ± 0.013	0.065 ± 0.015	0.1 ± 0.008

Table 3

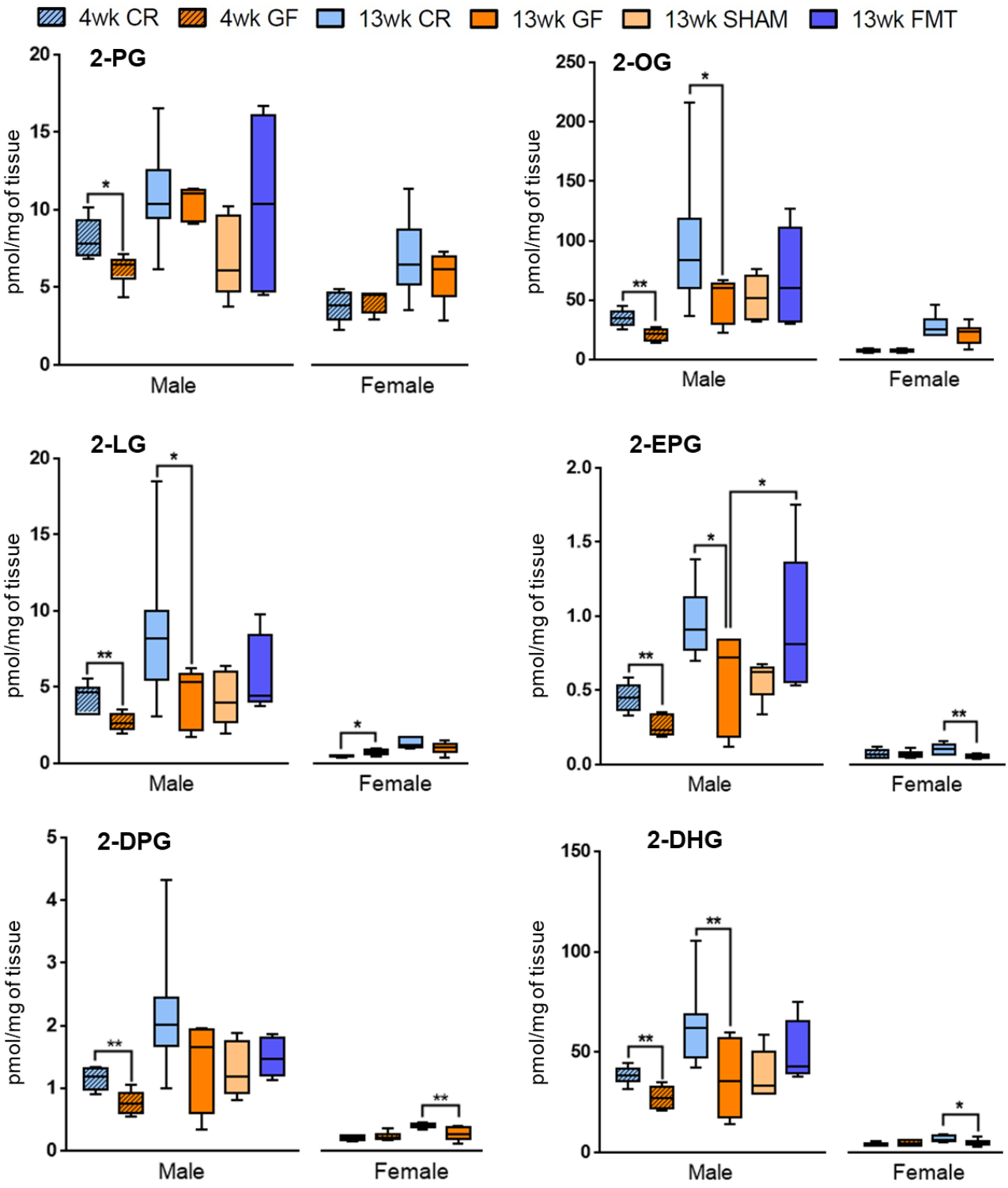

4wk CR
4wk GF
13wk CR
13wk GF
13wk SHAM
13wk FMT
2-PG
2-OG
2-LG
2-EPG
2-DPG
2-DHG
pmol/mg of tissue
Male
Female

Fig. 4

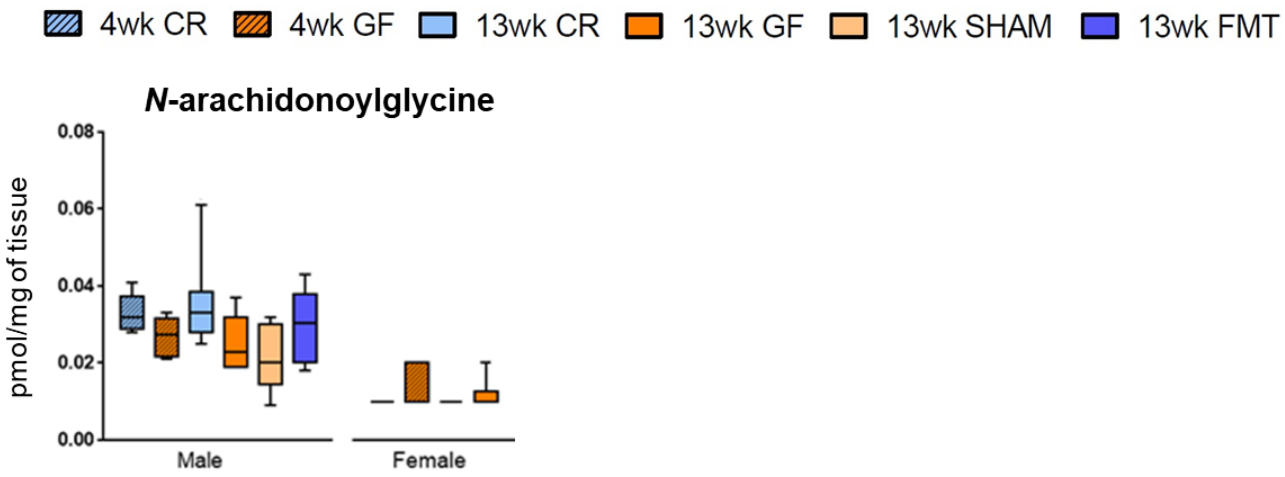

4wk CR
4wk GF
13wk CR
13wk GF
13wk SHAM
13wk FMT
N-arachidonoylglycine
pmol/mg of tissue
0.08
0.06
0.04
0.02
0.00
Male
Female

Fig. 5

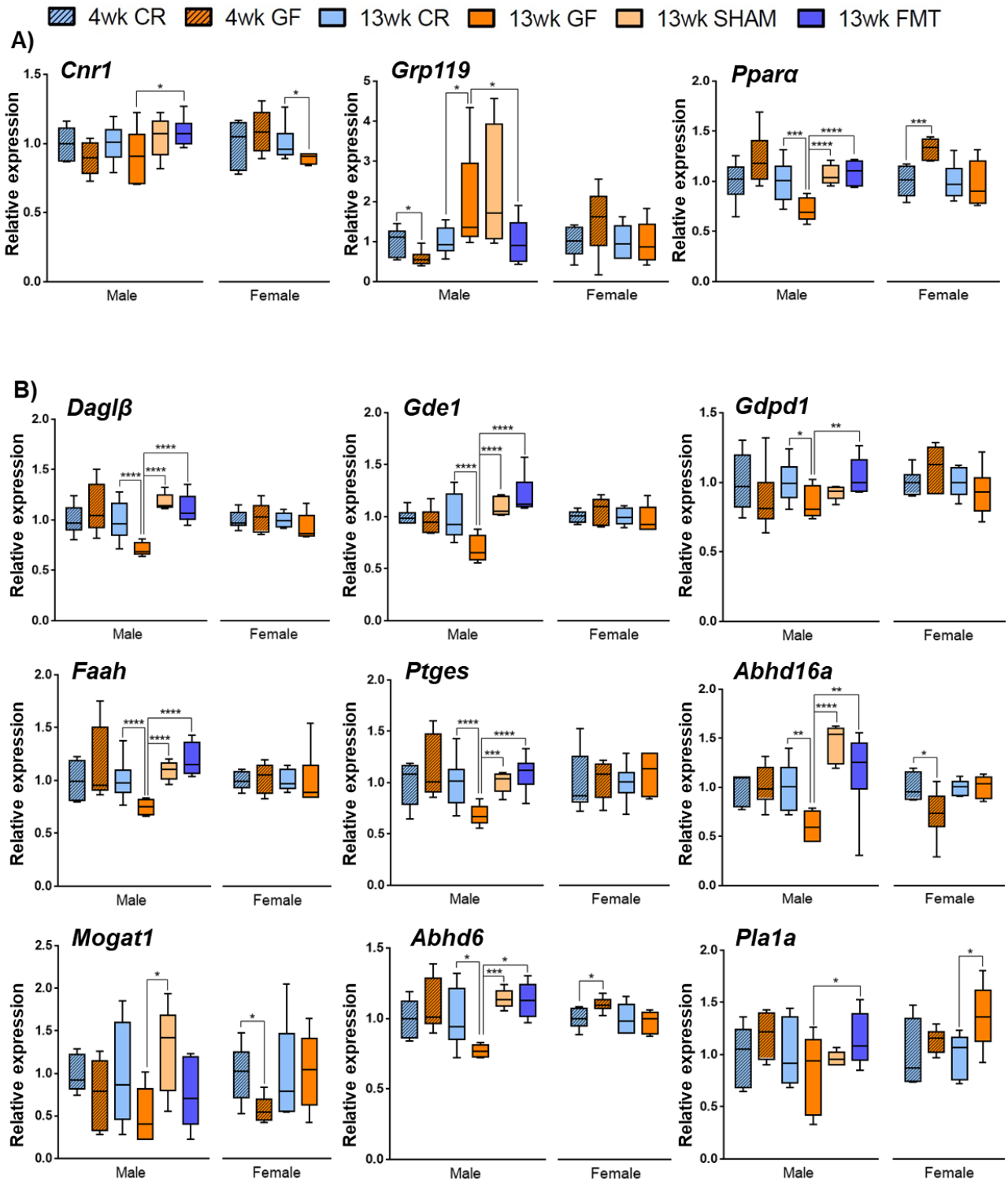

4wk CR
4wk GF
13wk CR
13wk GF
13wk SHAM
13wk FMT
A)
Cnr1
Grp119
Pparα
B)
Daglβ
Gde1
Gdpd1
Faah
Ptges
Abhd16a
Mogat1
Abhd6
Pla1a
Relative expression
Male
Female

Fig. 6