



Article

Exploring the Impact of the Microbiome on Neuroactive Steroid Levels in Germ-Free Animals

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Abstract: Steroid hormones are essential biomolecules for human physiology as they modulate the endocrine system, nervous function and behaviour. Recent studies have shown that the gut microbiota is directly involved in the production and metabolism of steroid hormones in the periphery. However, the influence of the gut microbiota on levels of steroids acting and present in the brain (i.e., neuroactive steroids) is not fully understood. Therefore, using liquid chromatography–tandem mass spectrometry, we assessed the levels of several neuroactive steroids in various brain areas and the plasma of germ-free (GF) male mice and conventionally colonized controls. The data obtained indicate an increase in allopregnanolone levels associated with a decrease in those of 5 α -androstane-3 α , 17 β -diol (3 α -diol) in the plasma of GF mice. Moreover, an increase of dihydroprogesterone and isoallopregnanolone in the hippocampus, cerebellum, and cerebral cortex was also reported. Changes in dihydrotestosterone and 3 α -diol levels were also observed in the hippocampus of GF mice. In addition, an increase in dehydroepiandrosterone was associated with a decrease in testosterone levels in the hypothalamus of GF mice. Our findings suggest that the absence of microbes affects the neuroactive steroids in the periphery and the brain, supporting the evidence of a microbiota-mediated modulation of neuroendocrine pathways involved in preserving host brain functioning.

Keywords: liquid chromatography–tandem mass spectrometry; hippocampus; cerebellum; cerebral cortex; hypothalamus; plasma; androgens; allopregnanolone



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1. Introduction

Information relating to the chemical composition of ingested food, the dynamic equilibrium of the microbial ecosystem and the physiological state of the gastrointestinal (GI) tract reaches the brain through the so-called microbiome–gut–brain axis (MGBA). This is a complex bi-directional set of neuronal, immune, endocrine signaling pathways and molecules allowing communication between the gastrointestinal tract and the central nervous system (CNS) [1]. The gut microbiota is a key orchestrator of gut–brain axis communication, as besides maintaining the intestinal epithelial barrier integrity and providing information to the immune system, gut microbes are able to produce a number of neurotransmitters as well as microbial-derived neuroactive products such as short-chain fatty acids, and to directly signal the peripheral and central nervous systems, and potentially modulate their function [2,3]. In the brain, physiological processes such as the stress response, blood circulation, and digestion as well as tissue functioning, cell proliferation, and organ maturation are finely tuned by the combined action of the nervous and the endocrine systems, which

utilize neurotransmitters and steroid hormones to exchange information among the different brain regions and with the peripheral organs [4]. Recent studies have shown that the gut microbiota is capable of influencing the production of glucocorticoid hormones from adrenal glands [1,4,5]. Additionally, a role of sex steroids synthesized from the gonads (i.e., androgens, estrogens and progesterone) has also been ascertained. Indeed, sex differences have been reported in the composition of the gut microbiota, with specific phyla, families and genera variances occurring with clear effects of gonadectomy and steroid hormone replacement on gut bacteria in rodents [6–15]. Furthermore, behaviour usually linked to estrous cycle stage such as visceral pain is not evident in germ-free (GF) mice [13], hence an appropriate microbiota colonization is necessary for appropriate functioning of the endocrine and nervous systems. This interaction of steroid hormones and the microbiome is also evident in human studies where sex differences have been noted in gut microbiota composition and diversity [14–16]. The gut microbiota itself also influences sex steroid levels both in rodents and humans [17–20]. Both human and animal studies highlight the reciprocal connections between sex steroid hormones (i.e., steroids synthesized by peripheral glands) and gut microbiota [6–11]. Interestingly, sex steroids are not only synthesized by gonadal glands but also within the nervous system (i.e., neurosteroids). Indeed, precursors of these steroids, such as pregnenolone (PREG) and dehydroepiandrosterone (DHEA), as well as the sex steroids themselves, such as progesterone (PROG), testosterone (T) and their metabolites, dihydroprogesterone (DHP), allopregnanolone (ALLO), isoallopregnanolone (ISOALLO) and dihydrotestosterone (DHT), 5 α -androstane-3 α , 17 β -diol (3 α -diol) and 17 β -estradiol (17 β -E) have been identified in different brain regions [21,22]. Both steroid hormones and neurosteroids, which are both found in the nervous system, are collectively referred to as neuroactive steroids and are important physiological regulators of nervous system functioning [23]. In particular, DHP controls reproductive functions, as well as glutamatergic and GABAergic neurotransmission [24], whereas isoallopregnanolone influences the lipid bilayer model system containing cholesterol [25,26]. In the brain, androgen molecules have been shown to regulate dendritic spine maturation [27,28], behaviour [29,30], neurite growth [31], neurogenesis and neuronal survival [32], apoptosis [33] and catecholamine production [34]. To date, only one study, performed in male animals, has focused on the possible influence of the gut microbiota on neuroactive steroid levels. Indeed, as recently observed, specific pathogen-free (SPF) male mice displayed differential levels of neuroactive steroids in specific brain areas [35]. Nevertheless, GF animals, which are born and raised without any microorganisms, represent an invaluable tool for understanding the role of microbiota in modulating brain development and behaviour via the gut–brain axis [1,36–38]. Therefore, the current study investigates, for the first time, the impact of a complete lack of microbiome on the concentration of neuroactive steroids in both the periphery and the central nervous system of GF male mice [36], setting the base for understanding the biomolecular mechanisms behind the microbial-dependent modulation of the neuroendocrine system, with important implications for brain function and behavioral phenotypes.

2. Results

The levels of different neuroactive steroids were assessed in the plasma and different brain regions of male GF mice and compared with those observed in conventionally colonized (CV) mice. Quantitative analysis of all steroids was achieved based on calibration curves; each steroid concentration was calculated as pg/sample. Total pg/sample values were normalized by tissue weight (i.e., μ L for plasma and mg for hippocampus, cerebellum, cerebral cortex and hypothalamus).

2.1. Assessment of Neuroactive Steroid Levels in Plasma

LC–MS/MS analysis showed a significant increase in ALLO concentrations ($p = 0.043$), associated with a decrease in the levels of 3 α -diol ($p = 0.028$) in the plasma of GF in comparison to CV mice (Figure 1). No significant changes in the other neuroactive steroids

assessed (i.e., PREG, PROG, DHP, ISOALLO, DHEA, T, DHT and 17β -E) were observed, even if a tendency to towards a decrease that did not reach the statistical significance was reported in the case of T in GF male mice.

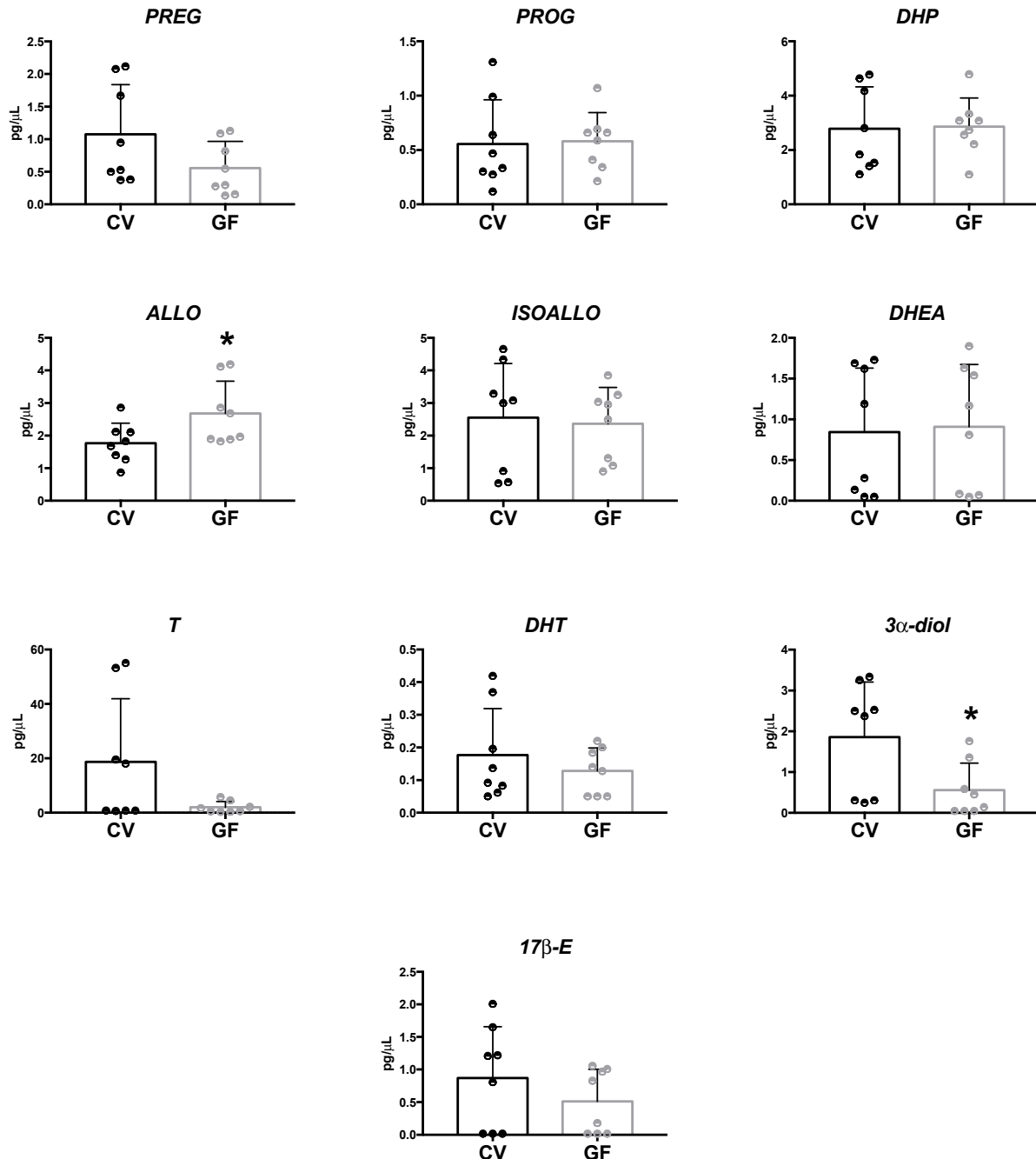


Figure 1. Levels of neuroactive steroids in the plasma of germ-free (GF) and conventional (CV) male mice. Data are expressed as $\text{pg}/\mu\text{L} \pm \text{SD}$, $n = 8$ for each group. Unpaired Student's *t*-test analysis: * $p < 0.05$ vs. CV mice.

2.2. Assessment of Neuroactive Steroid Levels in Brain Areas

2.2.1. Hippocampus

In the hippocampus, LC–MS/MS analysis evidenced a significant increase of DHP ($p = 0.007$), ISOALLO ($p = 0.003$), and 3α -diol ($p = 0.033$) levels in GF compared to CV mice (Figure 2). In the same brain region, DHT concentrations were found to be significantly reduced ($p = 0.049$) in GF compared to CV mice (Figure 2). Similar to what was noted

in the plasma of GF animals, the levels of PREG, PROG, DHEA, T and 17β -E present in the hippocampus of GF animals were not significantly different vs. those reported in CV animals (Figure 2). Some tendencies towards a decrease (i.e., DHEA and T) and towards an increase (i.e., ALLO) that did not reach statistical significance were reported in GF male mice (Figure 2).

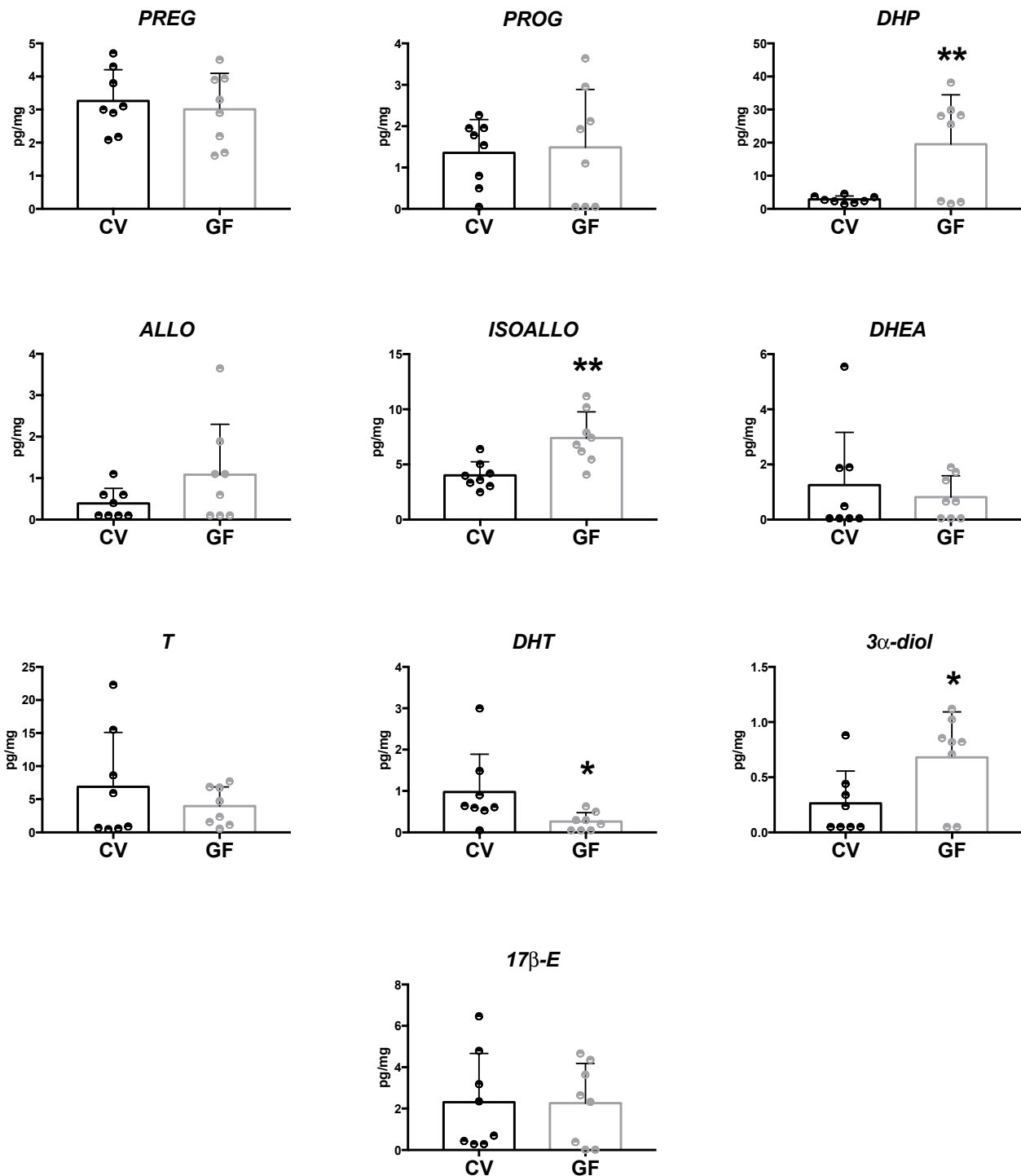


Figure 2. Levels of neuroactive steroids in the hippocampus of germ-free (GF) and conventional (CV) male mice. Data are expressed as pg/mg \pm SD, $n = 8$ for each group. Unpaired student's t -test analysis: * $p < 0.05$ ** $p < 0.01$ vs. CV mice.

2.2.2. Cerebellum and Cerebral Cortex

The LC–MS/MS analysis of neuroactive steroids showed an increase of DHP and ISOALLO levels in the cerebellum ($p = 0.028$; $p = 0.0003$, respectively; Figure 3) and cerebral cortex ($p = 0.010$; $p < 0.0001$, respectively; Figure 4) of GF compared with CV mice. However, in these two brain areas, the concentrations of the other neuroactive steroids were comparable between the GF and CV mice. Even if a tendency towards an increase was reported in the cerebral cortex of GF male mice for 17β -E and 3α -diol, this did not reach statistical significance.

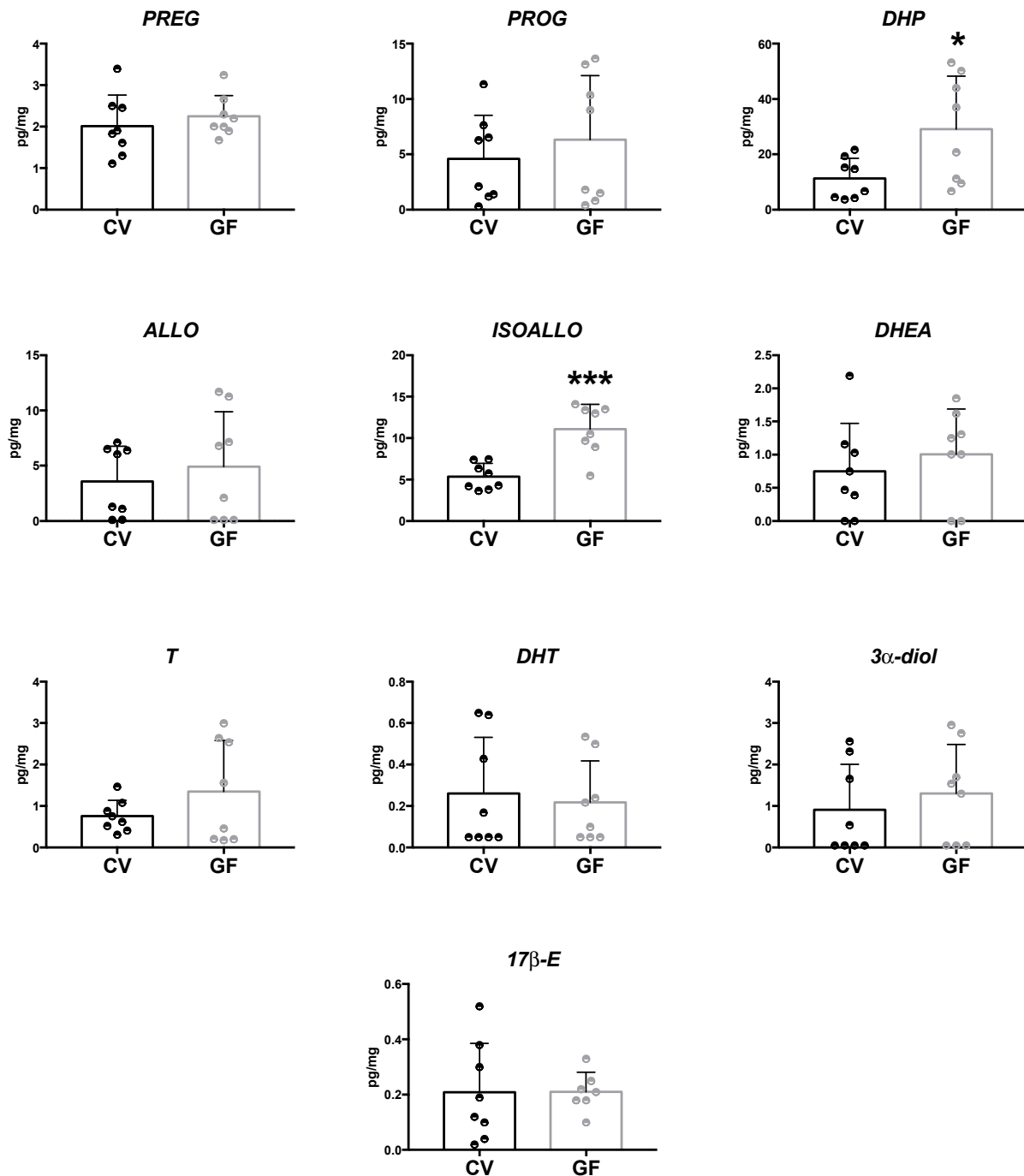


Figure 3. Levels of neuroactive steroids in the cerebellum of germ-free (GF) and conventional (CV) male mice. Data are expressed as pg/mg \pm SD, n = 8 for each group. Unpaired student's *t*-test analysis: * $p < 0.05$ *** $p < 0.001$ vs. CV mice.

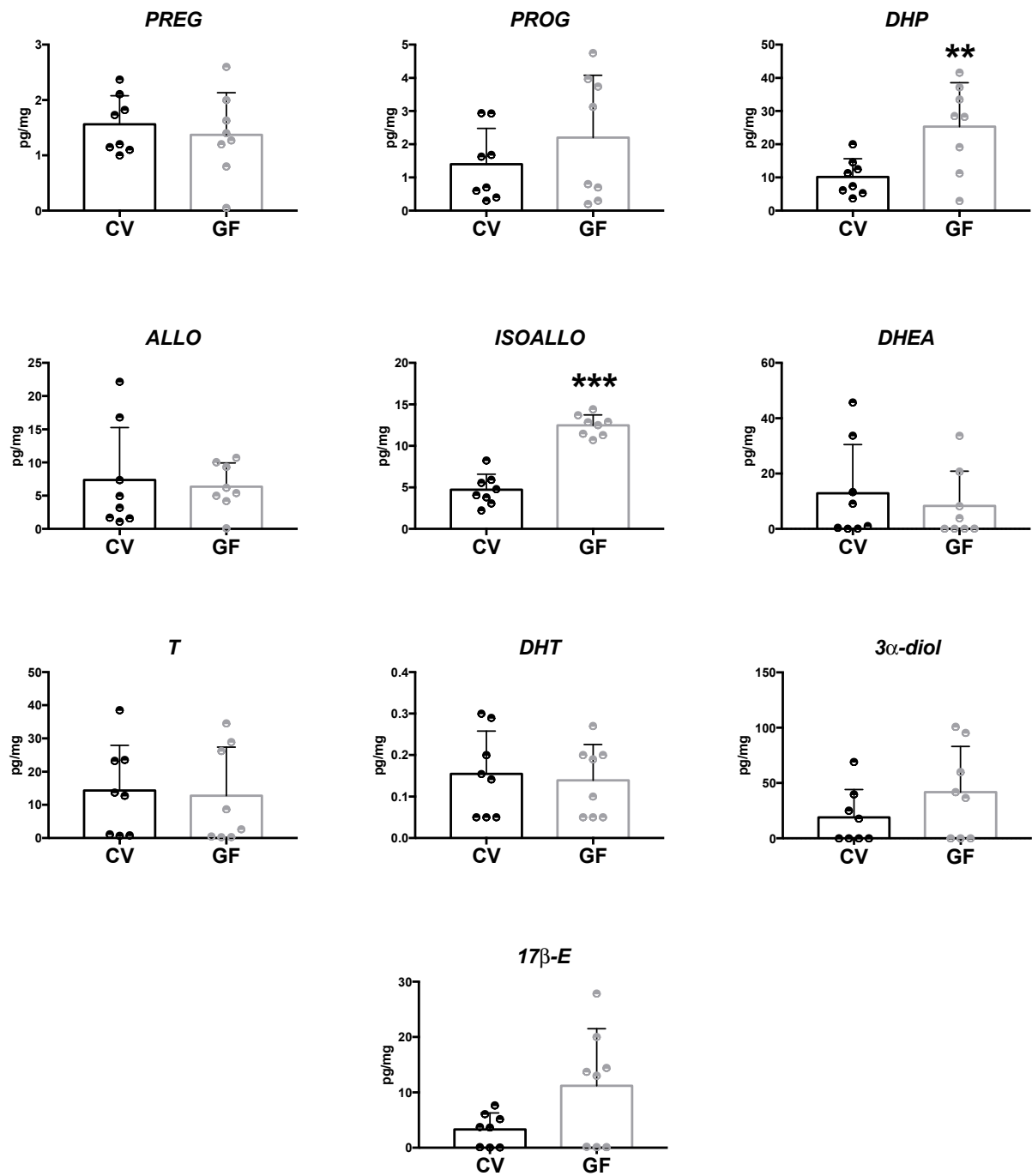


Figure 4. Levels of neuroactive steroids in the cerebral cortex of germ-free (GF) and conventional (CV) male mice. Data are expressed as pg/mg \pm SD, $n = 8$ for each group. Unpaired student's t -test analysis: ** $p < 0.01$ *** $p < 0.001$ vs. CV mice.

2.2.3. Hypothalamus

As showed in Figure 5, in the hypothalamus of GF animals, the LC–MS/MS analysis revealed a significant increase in DHEA concentration ($p = 0.027$), whereas the levels of T were found to be significantly reduced ($p = 0.040$). The concentrations of the other neuroactive steroids assessed were similar in both the GF and CV mice (Figure 5). In the cases of PREG and ALLO, a tendency towards an increase was reported in the hypothalamus of GF male mice that, however, did not reach statistical significance.

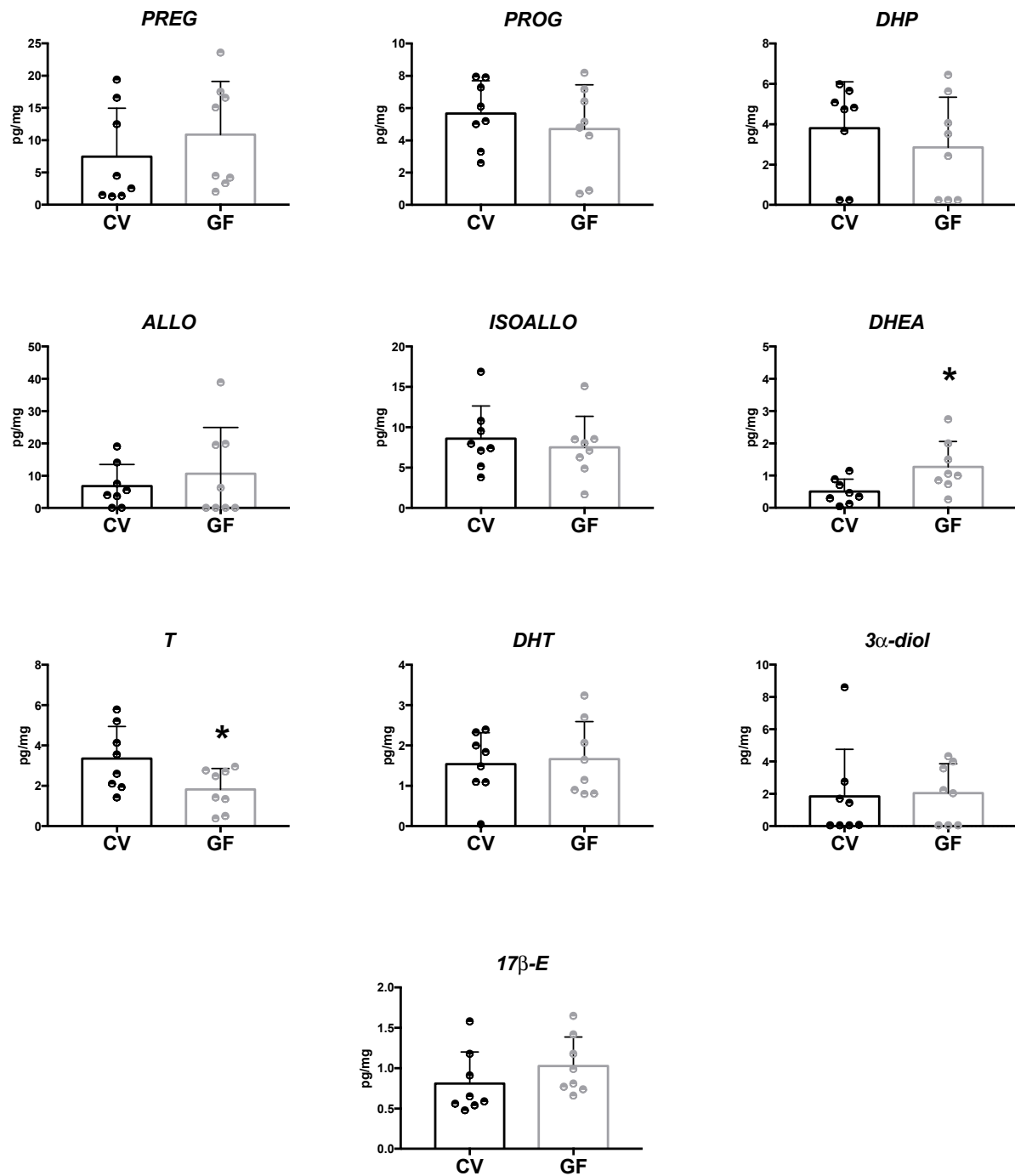


Figure 5. Levels of neuroactive steroids in the hypothalamus of germ-free (GF) and conventional (CV) male mice. Data are expressed as pg/mg \pm SD, n = 8 for each group. Unpaired student's *t*-test analysis: * $p < 0.05$ vs. CV mice.

3. Discussion

The complexity of the bidirectional communication between the gut microbiota, the GI system, and the brain mediated by the multiple signaling pathways and mechanisms of the gut–brain axis is beginning to be elucidated. One of the key pathways in this system is represented by the host–microbe interactions. The possibility of having animals raised without any microorganisms facilitates the investigation of the physiological units and biochemical processes by which such microbes finely tune the development and function of the GI tract and the brain. This study shows for the first time that in the absence of the microbiome, as in GF mice, the concentration of several neuroactive steroids both in

plasma and in the brain is altered compared with that of conventionally colonized mice. Our findings highlight a critical role of the microbiome in modulating these important physiological regulators of the nervous system, supporting the existence of a microbial-neuroendocrine signature in preserving brain integrity and function.

Interestingly, two main aspects emerged from these observations. Firstly, the gut microbiota influenced both plasma and CNS levels of neuroactive steroids, but these two compartments appeared to be differentially influenced, as the alterations noted in the plasma differed from those occurring in the brain. Thus, this finding suggests a divergent impact of the gut microbiota on peripheral steroidogenesis and neurosteroidogenesis. This is in line with observations reported in several physiopathological experimental models indicating the divergence of these two pools of steroids [21,39,40].

Secondly, in certain brain regions of GF animals, namely the hippocampus, cerebellum and cerebral cortex, the levels of the same neuroactive steroids were similarly altered. Indeed, in these three brain regions an increase in DHP and its metabolite, ISOALLO, was noted. This finding suggests that these two neuroactive steroids may represent a common signal for several brain areas in the MGBA.

On the other hand, depending on the brain areas considered, specific changes in neuroactive steroids also occurred. Indeed, in the hippocampus of GF mice a decrease in DHT and an increase in 3α -diol levels were observed, while in the hypothalamus, we reported an increase in DHEA and a decrease in T. Thus, there is not only a common pattern of changes (i.e., altered DHP and ISOALLO levels), but also specific changes of neuroactive steroid levels depending on the brain regions considered. DHEA and T levels have been reported to be altered in the hypothalamus of SPF animals as well; however, in this case an opposite pattern was observed [35]. Indeed, in SPF animals, a decrease in DHEA and an increase in T was reported [35], suggesting that different microbiota populations evoked different effects on the levels of these neuroactive steroids. In this context, it is important to highlight that in the hypothalamus of SPF male animals, DHEA levels were positively correlated with *Calditrichaeota* phylum [35].

All these neuroactive steroids exert a variety of physiological effects on the nervous system [39,40]. In this context, it is important to highlight that neuroactive steroids interact with different receptors. Indeed, while DHP—like its precursor PROG—is able to bind with the PROG receptor, ISOALLO—much like ALLO (i.e., another metabolite of PROG)—interacts with the GABA-A receptor. However, ISOALLO, in contrast to ALLO—which is a potent ligand of the GABA-A receptor [41,42]—does not bind directly to this neurotransmitter receptor [43], but instead antagonizes the effect of ALLO on the GABA-A receptor [44,45]. In addition, T and DHT, even if with different affinities, bind to the androgen receptor (AR), while their metabolite 3α -diol interacts with the GABA-A receptor. The mechanism of action of DHEA in the nervous system has not yet been fully characterized. Observations so far obtained show modulatory effects of this neuroactive steroid on membrane receptors, such as GABA-A, NMDA and sigma 1 receptors [46–49], while others suggest interactions with AR and its upregulation [50–53]. In this context, it is interesting to note that gut microbiota disturbances alter the expression of GABA-A [54,55] and NMDA receptors [56] in the rodent brain.

A further interesting link in the context of the gut microbiota–brain axis may be provided by the finding that chronic treatment with finasteride (i.e., an inhibitor of steroidogenic enzyme 5 α -reductase that converts PROG and T into their metabolites) induced changes in the gut microbiota populations of male rats (i.e., an increase in *Bacteroidetes* phylum and in the *Prevotellaceae* family) [57] and in post-finasteride patients [58]. In addition, similarly to what we have reported in the cerebellum of GF animals, an increase in DHP and ISOALLO also occurred in the cerebellum of finasteride-treated rats [59]. A possible hypothesis for this increase in the cerebellum of GF animals could be an increase in the gene expression of the enzyme 5 α -reductase, as we previously reported in finasteride-treated rats [59].

Altogether, these observations show for the first time that also neuroactive steroids in brain areas, like steroid hormones in the periphery [17–20], are affected by the gut microbiota population. Therefore, these findings may suggest that important physiological regulators of nervous function, such as neuroactive steroids, represent another molecular signal in the context of the MGBA and potential markers of MGBA-dependent psychiatric disorders. Future experiments will be needed to consolidate this important link and to evaluate the biomolecular mechanisms involved.

4. Materials and Methods

4.1. Animals

C57/Bl6 mice were purchased from Taconic (Hudson, NY, USA), and were bred as GF or conventionally raised in the animal facility of the Bioscience building, University College Cork, Cork Ireland. Breeding was performed according to supplier guidelines, and we used male offspring from F1-generation. GF male mice were housed 4 per cage in individually ventilated cages (area: 420 cm². Arrownight, UK), sex- and age-matched conventional mice were housed 4 mice/cage but in standard cages (area: 330 cm². NKP isotech, UK). GF and conventional mice were kept at the same temperature (21 ± 1 °C) and humidity (55–60%) conditions on a 12 h light/dark cycle and maintained on an ad libitum autoclaved water and autoclaved diet (pellet, Special Diet Services, Product code 801010).

4.2. Tissue Collection

Male GF (12 ± 1 weeks old; n = 8) and age- and sex- matched conventional mice (n = 8) were culled by decapitation and trunk blood was immediately collected in a K2 EDTA lavender-top vacutainer (BD Life Sciences). Blood was centrifuged at 3500 × g for 15 min at 4 °C, and plasma was collected in pre-weighed 1.5 Eppendorf tubes and stored at –80 °C until analysis. Brains were immediately collected and placed in petri dishes containing wet ice. For each brain, regions such as the hypothalamus, hippocampus, cerebellum, or cerebral cortex were manually dissected and placed in pre-weighed 1.5 Eppendorf tubes. Brain regions were snap frozen at –80 °C and kept in these conditions until analysis.

4.3. Reagents and Chemicals

Pregnenolone (PREG), pregnenolone-20,21-¹³C₂-16,16 D₂ (¹³C₂ D₂-PREG), progesterone (PROG), progesterone-2,3,4,20,25-¹³C₅ (¹³C₅-PROG), 17β-Estradiol (17β-E), 17β-Estradiol-2,3,4-¹³C₃ (¹³C₃-17β-E) dihydroprogesterone (DHP), allopregnanolone (ALLO), isoallopregnanolone (ISOALLO), testosterone (T), dihydrotestosterone (DHT), 5α-androstane-3α,17β-diol (3α-diol) and dehydroepiandrosterone (DHEA) were purchased from Merck Life Science, Italy. Acetonitrile, acetic acid, formic acid, methanol, 2-propanol and water were HPLC grade (Merck Life Science, Milano, Italy).

4.4. Liquid Chromatography–Tandem Mass Spectrometry Analysis

For the quantitative analysis of neuroactive steroids, brain tissues and plasma samples were extracted and purified as previously described [21,60–62]. ¹³C₃-17β-E (2 ng/sample), ¹³C₅-PROG (0.4 ng/sample) and ¹³C₂ D₂-PREG (10 ng/sample) were used as internal standards. For quantitative analysis of steroids, cerebral cortex, hypothalamus, hippocampus, cerebellum and plasma were collected, and internal standards were added. Tissue samples were homogenized using a Tissue Lyser (Qiagen, Italy), in ice-cold MeOH/acetic acid 1%. All tissues and plasma were purified by organic phase extraction, as previously described [21,60–62]. The analysis was conducted by liquid chromatography (LC) using an LC Pump Plus and Surveyor Autosampler Plus (Thermo Fisher Scientific, San Jose, CA, USA) with a linear ion trap-mass spectrometer (LTQ, Thermo Fisher Scientific, San Jose, CA, USA) operating in positive atmospheric pressure chemical ionization (APCI+). The chromatographic separation was achieved with a Hypersil Gold column C18 (100 × 2.1 mm, 3 μm; ThermoFisher Scientific) maintained at 40 °C. The mobile phases consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B).

The gradient elution was as follows: 0–1.50 min 70% A, 30% B; 1.50–2.00 min 55% A, 45% B; 2.00–3.00 min. 55% A, 45% B; 3.00–35.00 min. linear gradient to 36% A, 64% B; 35.00–40.00 min. 25% A, 75% B; 41.00–45.00 min. 1% A, 99% B; 45.00–45.20 min. 70% A, 30% B and 45.40–55.00 min equilibrated with 70% A and 30% B. A sample of 25 µL was injected at a flowrate of 0.250 mL/min. The divert valve was set at 0–8 min to waste, 8–45 min to source and 45–55 min to waste. The injector needle was washed with MeOH/Water 1/1 (*v/v*). Quantitative analysis was performed on the basis of calibration curves prepared and analyzed using standards. LC–MS/MS peaks were appraised using the software Excalibur® release 2.0 SR2 (Thermo Fisher Scientific, San Jose, CA, USA). Quantitative analysis of all steroids was achieved based on freshly prepared calibration curves. Detection limits were 0.02 pg/µL or pg/mg for T and 17β-E, 0.05 pg/µL or pg/mg for PREG, PROG, 3α-diol, DHEA, DHT; 0.1 pg/µL or pg/mg for ALLO and ISOALLO; 0.25 pg/µL or pg/mg for DHP.

4.5. Statistical Analysis

Data for LC–MS/MS ($n = 8$ per experimental group) were analyzed by unpaired Student's *t*-test, after checking for normal distribution with the Kolmogorov–Smirnov test. $p < 0.05$ was considered significant. Analyses were performed using Prism, version 7.0a (GraphPad Software Inc., San Diego, CA, USA).

Author Contributions: S.M.O. and R.C.M. designed the experiments; S.D., L.C., V.C. and J.M.L. performed the experiments; S.D., L.C. and D.C. analyzed the data; R.C.M., S.D., V.C., S.M.O. and S.G. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal-related experiments were approved by the Animal Experimentation Ethics Committee of University College Cork and Health Products Regulatory Authority (HPRA) before beginning this study (n. AE19130/P047). All experimentation was carried out in accordance with European and National Directives (2010/63/EU and SI No 543 of 2012, respectively).

Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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