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Treatment of male rats with finasteride, an inhibitor of 5alpha-reductase enzyme, induces long-lasting effects on depressive-like behavior, hippocampal neurogenesis, neuroinflammation and gut microbiota composition

Silvia Diviccaro[a], Silvia Giatti[a], Francesca Borgo[b], Matteo Barcella[b], Elisa Borghi[b], José Luis Trejo[c], Luis Miguel Garcia-Segura[c,d], Roberto Cosimo Melcangi[a,*]

[a] Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, Italy
[b] Dipartimento di Scienze della Salute, Università degli Studi di Milano, Milan, Italy
[c] Instituto Cajal, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain
[d] Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES), Instituto de Salud Carlos III, Madrid, Spain

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ABSTRACT

Persistent alteration of plasma neuroactive steroid levels associated with major depression has been recently reported in men after the suspension of the treatment for androgenetic alopecia with finasteride, an inhibitor of the enzyme 5alpha-reductase. Observations in male rats confirmed persistent alterations in neuroactive steroid levels also in the brain. In the present study, we have ascertained possible effects on depressive-like behavior, neurogenesis, gliosis, neuroinflammation and gut microbiota in male rats after subchronic treatment for 20 days with finasteride and after one month of its withdrawal. At the end of treatment there was an increase in the number of pH3 immunoreactive cells in the subgranular zone of the dentate gyrus together with an increase in the mRNA levels of TNF-α in the hippocampus. By one month after the end of finasteride treatment, rats showed depressive-like behavior coupled with a decrease in the number of pH3 immunoreactive cells in the subgranular zone of the dentate gyrus, a decrease in granule cell density in the granule cell layer and an increase in the number of GFAP immunoreactive astrocytes in the dentate gyrus. Finally, alteration of gut microbiota (i.e., an increase in *Bacteroidetes* phylum and in *Prevotellaceae* family at the end of the treatment and a decrease in *Ruminococcaceae* family, *Oscillospira* and *Lachnospira* genus at the end of the withdrawal period) was detected.

In conclusion, finasteride treatment in male rats has long term effects on depressive-like behavior, hippocampal neurogenesis and neuroinflammation and gut microbiota composition.

1. Introduction

Neuroactive steroids are those steroids that modulate the function of the nervous system. They are important physiological neuromodulators and include steroids synthesized in the peripheral glands as well as those directly produced by the nervous tissue. Neuroactive steroids are involved in the neuroendocrine control of reproduction and regulate synaptic plasticity, neuronal and glial morphology, adult neurogenesis, myelination and cognition (Galea, 2008; Melcangi et al., 2016). Accordingly, psychiatric disorders, such as depression and anxiety, are associated with altered levels of neuroactive steroids, and particularly of 5α-reduced metabolites of testosterone and progesterone, in plasma and/or cerebrospinal fluid (CSF) (Backstrom et al., 2014; Schule et al., 2014).

Finasteride, an inhibitor of the enzyme 5α-reductase (5α-R), blocks the conversion of testosterone and progesterone, into their 5α-reduced metabolites (dihydrotestosterone, DHT, and dihydroprogesterone, DHP, respectively). For this reason, this drug is used for the treatment of benign prostatic hyperplasia (BPH) and androgenetic alopecia (AGA). Indeed, these disorders are associated with high levels of DHT in prostate and in hair follicles of BPH and AGA patients, respectively. By inhibiting 5α-R activity, finasteride reduces DHT levels in prostate and in hair follicles and improve the conditions of BPH and AGA (Traish et al., 2015).

Despite of the wide therapeutic use of finasteride in humans, its effects in the nervous system have been poorly explored. That is

* Corresponding author at: Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Via Balzaretti 9, 20133 Milano, Italy.
E-mail address: roberto.melcangi@unimi.it (R.C. Melcangi).

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particularly important, because a subset of men taking finasteride for AGA show during the treatment a wide symptomatology including depression, erectile dysfunction, endocrine alterations and musculoskeletal manifestations (Giatti et al., 2018). Moreover, as self-reported by the patients (Altomare and Capella, 2002; Irwig, 2012; Rahimi-Ardabili et al., 2006; Traish et al., 2015) and as recently ascertained by two different clinical studies, these important side effects may persist even after discontinuation of the treatment (Basaria et al., 2016; Melcangi et al., 2017). Thus, these patients are affected by the so called post-finasteride syndrome (PFS). In particular, as reported by Basaria and collaborators, PFS patients have impaired sexual function as well as high depression scores (Basaria et al., 2016). In sixteen PFS male patients, we recently observed that 1) all patients showed erectile dysfunction, 2) four of them had altered peripheral neurogenic control of erection and 3) eight of them suffered from a DSM-IV major depressive disorder (Melcangi et al., 2017).

In addition, in plasma and CSF of PFS patients, suspension of drug treatment did not only lead, as expected, to an alteration of 5α-reduced metabolites of testosterone and progesterone, but also to a global alteration of neuroactive steroid levels (Caruso et al., 2015; Melcangi et al., 2013, 2017). Interestingly, observations obtained in male rats indicate that finasteride treatment and its suspension not only alters the levels of neuroactive steroids in plasma and CSF, but also in the brain tissue. However, it is important to note that the effects in plasma or CSF are different in comparison to what observed in brain areas. For instance, subchronic treatment with finasteride (i.e., for 20 days) induced only an increase of progesterone in the hippocampus, while in plasma only an increase in isopregnanolone (i.e., a metabolite of DHP) associated with a decrease in DHT were observed (Giatti et al., 2016). At the withdrawal (i.e., after one month of suspension) a decrease of pregnenolone (i.e., the first neuroactive steroid synthesize from cholesterol) and progesterone associated with an increase of DHP were observed in the hippocampus, while a decrease in progesterone, tetrahydroprogesterone, THP (i.e., a metabolite of DHP), DHT and 3α-diol (i.e., a metabolite of DHT) was observed in plasma (Giatti et al., 2016). Interestingly, neither subchronic treatment with finasteride nor its withdrawal were able to affect the levels of DHT in the hippocampus (Giatti et al., 2016). In addition, the brain changes in neuroactive steroid levels were coupled with altered expression of their receptors, suggesting an altered brain steroid signaling (Giatti et al., 2016).

Depression is a multifaceted psychiatric condition, where genetic susceptibility and environmental factors crosstalk to generate a distressing condition. As reported in humans and in animal models that mimic this condition, depression is characterized by decreased content of neurotransmitters and neurotrophic factors, increased inflammation and gliosis, decreased neurogenesis and neuron survival and mitochondrial impairment (Blier, 2013; Masi and Brovedani, 2011).

Gut microbiota is an exclusive combination of different organisms, such as bacteria, viruses, archaea, protozoa and fungi that interact in a bidirectional way with the central nervous system forming the so call microbiome-gut-brain axis (Mayer, 2011; Sharon et al., 2016). Immune, neural, endocrine and metabolic pathways are included in this axis. Among them, steroid hormones have also an important role. Indeed, several observations indicate that steroid hormones influence gut microbiota niches (Tetel et al., 2018). For instance, gonadectomy or steroid treatment affect the composition of gut microbiota in rodents (Org et al., 2016). Several recent observations seem also to suggest a link between gut microbiota and development and/or manifestation of neuropsychiatric disorders, such as depression and anxiety (Borgo et al., 2017; Foster and McVey Neufeld, 2013). Indeed, microbiota composition is altered both in animal models and in patients with this symptomatology (Hoban et al., 2016; Kelly et al., 2016).

On this basis, our main hypothesis is that treatment with finasteride and/or its withdrawal in male rat may induce depressive-like behavior related with cellular and/or molecular alterations in hippocampus and/or gut microbiota composition. Therefore, in the present study we will explore: (1), whether subchronic treatment with this inhibitor of the enzyme 5α-R induces depressive-like behavior in male rats; (2), whether this alteration persists by 1 month after the end of the treatment and (3), whether finasteride treatment and/or withdrawal is associated with modifications in neurogenesis, gliosis and neuroinflammatory parameters in the hippocampus and with changes in gut microbiota.

2. Materials and methods

2.1. Animals and treatments

Male Sprague Dawley rats (250–275 g at arrival, Janvier Labs, Le Genest- Saint-Isle, France) were housed in the animal care facility of the Instituto Cajal, C.S.I.C., Madrid, Spain. All animals were kept in standard rat cages with food and tap water available *ad libitum* and under controlled humidity and temperature. The rats were acclimated to the new environment for 7 days before being randomly assigned to one of the experimental groups described below. All procedures for handling and killing the animals used in the study were in accordance with the European Commission guidelines (86/609/CEE and 2010/63/UE) and Spanish regulation (R.D. 53/2013) on the protection of animals for experimental use and were approved by our institutional animal care and use committee (Comité de Experimentaciòn Animal del Instituto Cajal) and the Consejeria del Medio Ambiente y Territorio (Comunidad de Madrid, Ref. PROEX 200/14).

Finasteride (3 mg/kg/day; Sigma-Aldrich, Italy) was dissolved in a vehicle solution of corn oil and ethanol (7% v/v). Solutions were injected subcutaneously, at a volume of 100 μL/day. We used a group of 40 rats in the present study. The whole cohort was divided in two main experimental groups, referred to after treatment (i.e. 24 h after the last injection; n = 20) and 1 month after withdrawal (i.e. 1 month after the last injection; n = 20), respectively. In each experimental group, the rats were randomly assigned to vehicle-treated rat group (Control, C) *vs* finasteride-treated group (Finasteride, F). The vehicle and drug solutions were administrated daily for 20 days.

2.2. Behavioral test: Forced swim test

Forced swim test is a classic paradigm of despair which measures depressive behavior in rodents. Animals were introduced for 6 min per day in two consecutive days (i.e., pre-test session day 1 and test session day 2) into a methacrylate cylinder, a transparent plastic resistant material, containing water at a temperature of 23–24 °C. The water temperature (T) is very important: a lower T causes hyperactivity that could stress the animal while on the contrary a higher T could relax the animal (Jefferys and Funder, 1994). We have only analyzed the time of immobility of day 2, which is considered a measure of depressive-like behavior.

2.3. BrdU injection

The study of proliferation and survival of newborn adult hippocampal neurons was performed using the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) at a dose equimolecular of 50 mg/kg, as published elsewhere (Llorens-Martin et al., 2010). The experimental groups received one i.p. injection of BrdU 24 h before sacrifice.

2.4. Immunohistochemistry (IHC)

Rats were deeply anesthetized and then perfused through the left cardiac ventricle first with pre-warmed (37 °C) 0.9% NaCl, followed by 4% paraformaldehyde (pH 7.4; 37 °C). The brains were dissected out and post-fixed overnight at 4 °C in 4% paraformaldehyde and washed three times with 0.1 M phosphate buffer (PB), pH 7.4 at room temperature (RT). Coronal brain sections, 50 μm thick, were obtained by Leica VT1000S Vibratome (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry was carried out in free-floating sections under moderate shaking. For each primary antibody, sections were sampled one out of every six brain coronal slices. Phospho-Histone H3 (pH3) is a marker for cells undergoing mitosis. We performed double IHC for pH3 and BrdU to analyze cell division. For pH3/ BrdU double immunofluorescence staining, sections were pre-incubated for 25 min with 2 N HCl at RT to gather antigen unmasking. Then, sections were washed 5 times for 5 min in 0.1 M PB containing 1% Triton X-100 and 1% bovine serum albumin. Double immunohistochemistry was performed using a rabbit anti-pH3 antibody (Upstate ref. 06–570, 1: 500) and a mouse anti-BrdU antibody (Hybridoma Bank ref. G3G4, 1:500). Sections were incubated with the primary antibodies for 2 h at RT and additional 48 h at 4 °C. Then, sections were washed in PBS and incubated for 2 h at RT with Alexa-488 goat anti-rabbit for anti-pH3 detection (1:1000) and Alexa-594 donkey anti-mouse for anti-BrdU detection (1:1000). Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI, Calbiochem, 1:1000) for 10 min.

For diaminobenzidine (DAB) staining, endogenous peroxidases were blocked for 15 min at RT in a solution of 0.3% Triton X-100, 0.5% bovine serum albumin, 3% hydrogen peroxide and 50% methanol in 0.1 M PB. After several washes in PB, sections were incubated overnight with one of the following primary antibodies: mouse monoclonal antibody for glial fibrillary acidic protein (GFAP; diluted 1:1000; Clone GA5, Sigma-Aldrich), marker of reactive and resting astrocytes or rabbit polyclonal antibody for Ionized calcium binding adaptor molecule 1(Iba1; diluted 1:2000; Wako Chemical Industries, Japan), marker of microglia/macrophages. Sections were then rinsed in PB and incubated for 2 h at RT with the corresponding anti mouse or anti rabbit biotinylated secondary antibody (diluted 1:300; Pierce). After several washes in PB, sections were incubated for 90 min at RT with avidin-biotin-peroxidase complex (diluted 1:500; ImmunoPure ABC peroxidase staining kit). The reaction product was revealed by incubating the sections with 2 mg/mL DAB (Sigma-Aldrich) and 0.01% hydrogen peroxide in 0.1 M phosphate buffer. The DAB-stained sections were dehydrated in graded alcohols and xylenes, mounted on gelatinized slides, cover slipped, and examined with a Leica DMRB-E microscope.

2.5. Morphometric analysis

The number of BrdU or pH3 immunoreactive cells was assessed in the subgranular zone of the dentate gyrus. To obtain the total number of pH3 immunoreactive cells and the total number of BrdU cells per hippocampus, the number of cells in each section was added for each animal and multiplied by six, in accordance with the section sampling interval.

The number of cells immunoreactive for GFAP or Iba-1 was estimated with the optical disector method in the hilus of the dentate gyrus of the hippocampus using total section thickness for disector height and a counting frame of 217 × 162 μm. A total number of 60 counting frames were assessed per animal (20 for each slice) using a 40X objective.

2.6. Granule cell density analysis

DAPI stained sections were used for quantitative analysis of granule cell density in the granule cell layer of the hippocampus as described previously (Llorens-Martin et al., 2006). For each rat, Z-series stacks were made in four different areas of granule cell layer: one rostral stack, one medial stack and two caudal stack with a 63X objective. Other parameters set for Z-stack were as follows: six images for each Z-stack, zoom 3, 10 μm of z-series thickness, and a resolution 1024 × 1024 pixels. Granule cell density was performed counting each Z-stack in Image-J program.

2.7. Real time PCR

Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) mRNA levels were assessed in the hippocampus by quantitative real-time polymerase chain reaction (PCR). RNA from frozen tissue was extracted using Direct-zol™ MiniPrep kit (Zymo Research, Irvine, Calif., USA) following manufacturing protocol. RNA was quantified by NanoDrop™ 2000 (ThermoFisher scientific, Milano, Italy). TaqMan quantitative real-time PCR was performed by CFX96 real-time system (Bio-Rad Laboratories, Segrate, Italy). Samples were run in duplicate as multiplexed reactions with a normalizing internal control, 36B4 (Eurofins MWG- Operon, Milano, Italy) in 96-well formats using the iTaq™ Universal Probes One-Step Kit (Bio-Rad, Segrate, Italy). Specific TaqMan MGB probes and primers sequence were designed using Eurofins MWG-Operon (Milano, Italy) and were as follows: for IL-1β, forward, 5′-TGCAGGCTTCGAGATGAAC-3′ and reverse, 5′-GGGATTTT GTCGTTGCTTGTC-3′; for TNF-α, forward, 5′-CTTCTCATTCCTGCTCG TGG-3′ and reverse, 5′-TGATCTGAGTGTGAGGGTCTG-3′; for 36B4, forward, 5′-GGATGACTACCCAAAATGCTTC-3′ and reverse, 5′-TGGTG TTCTTGCCCATCAG -3′. All genes were normalized to 36B4.

2.8. Bacterial DNA extraction and 16S rRNA gene sequencing

Total bacterial DNA was extracted from 200 mg of stool samples using the Spin stool DNA kit (Stratec Molecular, Berlin, Germany), according to the manufacturer's instructions and amplified by PCR. 25 ng of DNA extracted and amplified was utilized to construct a sequencing library as previously described (Borghi et al., 2017). Library concentration and exact product size were measured using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) and an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA), respectively.

A pooled library was loaded on a MiSeq® v2 (500 cycle) Reagent cartridge for sequencing. Production of sequencing fragments was performed on Illumina MiSeq platform with a 250PE protocol. Samples were run in pool obtaining an average of 280k paired-end reads per sample.

Fastq files were checked for quality using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and data analysis was performed using QIIME2 suite. In particular, we performed sequencing quality control using the DADA2 pipeline that allow detecting and correcting Illumina amplicon sequence data, with the following parameters: –p-trunc-len-f 248, –p-trunc-len-r 240 and –p-trim-left 5. Quality passed sequences were grouped by DADA2 into sequence variants that are equivalent to Operational Taxonomic Units (OTUs) with 100% similarity threshold. Q2-feature-classifier, trained on the Greengenes 13_8 97% OTUs specifically on V3-V4 region, was used to perform taxonomic classification.

2.9. Statistical analysis

Kolmogorov-Smirnov test was preliminary applied in order to choose the appropriate statistical test to be used (i.e., parametric or non-parametric test). On the basis of this result, data of the behavioral analysis (n = 10 per experimental group) and real-time PCR analysis (n = 6 per experimental group) were analyzed by two-way analysis of variance (ANOVA), with treatment and time as two independent variables, followed by the Bonferroni post hoc test; number of immunoreactive cells (n = 4 per experimental group) were analyzed with the Mann-Whitney non-parametric test. For microbioma analysis (n = 10 per experimental group), sample biodiversity (i.e. α-diversity) was estimated according to different microbial diversity metrics including chao1, Shannon index, evenness, observed species and Faith's phylogenetic distance. Inter-sample diversity (i.e. β-diversity) was evaluated by using both weighted and unweighted Unifrac and Bray Curtis distance metrics. Principal Coordinates Analysis (PCoA) was

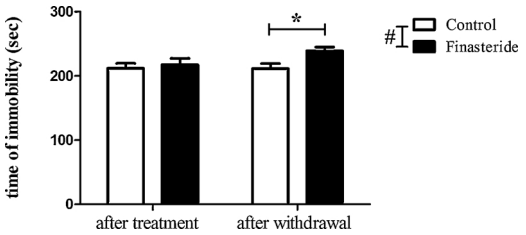

Fig. 1. Effect of finasteride on depressive-like behavior. Data represents the immobility time in the forced swim test in animals treated with vehicle (control) or finasteride at the end of the treatment and at one month after withdrawal. Data are represented as mean ± SEM, n = 10 for each experimental group. The effect of treatment, time and the interaction treatment by time were analyzed using two-way ANOVA (significance: # $p < 0.05$) followed by Bonferroni post hoc test (significance: * $p < 0.05$).

performed using build-in functions in QIIME2. Groups significance, according to experimental design, were calculated by Kruskal-Wallis test for alpha metrics vectors, whereas by PERMANOVA test for beta-metrics distance matrices. With the latter test is possible to evaluate whether distances between samples within a group are more similar to each other then they are to samples from the other groups. α and β metrics were evaluated by setting a sampling depth of 4354 according to DADA2 feature table summary. Relative abundance analysis of bacterial taxonomies between groups was evaluated using Mann-Whitney test ($p \leq 0.05$ as threshold).

3. Results

3.1. Forced swim test

Two-way ANOVA revealed a significant effect of treatment ($F = 4.22$, $p = 0.0474$) (Fig. 1). At the end of the treatment, no significant differences were detected in the immobility time in the Porsolt test between animals treated with vehicle or finasteride, neither in pre-test session (day 1, data not shown) or test session (day 2). However, as reported in Fig. 1, one month after withdrawal animals treated with finasteride showed a significantly higher immobility time in the Porsolt test compared with animals treated with vehicle during the test session (day 2) ($t = 2.427$, $p < 0.05$, Bonferroni post hoc test).

3.2. Number of BrdU and pH3 immunoreactive cells

Examples of DAPI staining of the dentate gyrus, and pH3 and BrdU immunoreactive cells in the subgranular zone of the dentate gyrus are shown in Fig. 2A–C. No significant differences were detected in the total number of BrdU immunoreactive cells in the subgranular zone between the animals treated with vehicle or finasteride, neither at the end of the treatment nor one month after withdrawal (data not shown). However, the number of pH3 immunoreactive cells was significantly increased by finasteride at the end of the treatment ($p = 0.0294$) and was significantly decreased ($p = 0.0284$) one month after finasteride withdrawal compared to vehicle injected animals (Fig. 2D). In addition, granule cell density in the granular cell layer (Fig. 2E) was significantly decreased in finasteride treated animals, but only after one month of finasteride withdrawal ($p = 0.0286$).

3.3. GFAP and Iba-1 immunoreactive cells

Examples of GFAP immunoreactive astrocytes in the hilus of the dentate gyrus are shown in Fig. 3A. Finasteride treatment increased the number of GFAP immunoreactive astrocytes in the hilus of the dentate gyrus compared to vehicle injected animals. This effect was detected only one month after finasteride withdrawal ($p = 0.0286$). One day after the end of the treatment only a tendency to increase ($p = 0.0571$) was observed (Fig. 3B). No significant differences were detected among the different experimental groups in the number of Iba-1 immunoreactive microglia in the hilus of the dentate gyrus (data not shown).

3.4. Gene expression of TNF-α and IL-1β

Two-way ANOVA revealed a significant effect of treatment ($F = 4.60$, $p = 0.0445$) on the mRNA levels of TNF-α of the hippocampus (Fig. 4A). In particular, Bonferroni post hoc test showed that mRNA levels were significantly increased at the end of the finasteride treatment compared to vehicle injected animals ($t = 3.253$, $p < 0.01$). On the contrary, mRNA levels of IL-1β were not significantly different (Fig. 4B).

3.5. Gut microbiota

The composition of rat gut microbiota in stool samples was characterized by next-generation sequencing using V3–V4 hyper-variable 16S rRNA genomic region. To determine changes in the intestinal microbiota due to finasteride treatment, we first performed α-diversity and β-diversity analyses within vehicle and finasteride groups on stools collected 24 h after 20-days treatment. We did not observe a statistically significant change in α-diversity (Fig. 5A) assessed by Chao1 ($p = 0.36$) and Shannon indices ($p = 0.14$). However, β-diversity analysis, revealed a significant diversity based on weighted ($p = 0.03$) and unweighted UniFrac distances ($p = 0.04$). At phylum level (Fig. 5B), the predominant bacterial taxa in stool samples were *Firmicutes* (C: 79.2 ± 5.9; F: 73.5 ± 7.7) and *Bacteroidetes* (C: 17.3 ± 5.5; F: 24.2 ± 7.8) followed by *Proteobacteria* (C: 1.4 ± 0.6; F: 1.6 ± 0.9) and *Verrucomicrobia* (C: 1.8 ± 0.4; F: 1.3 ± 0.9). At family level, *Bacteroidaceae* (C: 3.1 ± 0.2; F: 2.8 ± 0.9), *Prevotellaceae* (C: 1.3 ± 0.9; F: 3.6 ± 1.9), *Rikenellaceae* (C: 1.3 ± 0.8; F: 1.7 ± 0.6), *S24-7* (C: 10.6 ± 3.9; F: 11.8 ± 4.5), *Lactobacillaceae* (C: 26.7 ± 2.6; F: 25.0 ± 1.7), *Clostridiaceae* (C: 1.2 ± 0.7; F: 0.9 ± 0.5), *Lachnospiraceae* (C: 12.4 ± 4.2; F: 13.0 ± 4.5), *Ruminococcacea* (C: 16.4 ± 4.6; F: 18.3 ± 2.3) were the most abundant in both groups (Fig.5B). At genus level, *Bacteroides* (C: 2.3 ± 0.9; F: 2.8 ± 0.6), *Prevotella* (C: 3.6 ± 1.9; F: 5.1 ± 0.8), *S24-7* (C: 14.8 ± 4.5; F: 14.8 ± 4.5) and *Lactobacillus* (C: 20.0 ± 0.7; F: 19.2 ± 0.9) were the most representative bacteria (Fig. 5B). Finasteride group was significantly enriched in *Bacteroidetes* phylum ($p = 0.03$) and *Prevotellaceae* family ($p = 0.03$), suggesting that finasteride exposure could affect gut microbiota composition (Fig. 5B).

To evaluate possible long-lasting effects on microbiota after finasteride discontinuation, stools were collected one month after finasteride withdrawal and processed for microbiota analysis. Although α-diversity metrics showed no significant differences in bacterial richness between C and F groups (Shannon index: $p = 0.32$; Chao1: $p = 0.28$), we found significant changes in the microbial communities (weighted and unweighted UniFrac distance $p = 0.03$, $p = 0.03$, respectively) (Fig. 6A). The phylum *Firmicutes* (C: 76.2 ± 3.4; F: 72.7 ± 2.1) further decreased compared with basal values, whereas *Bacteroidetes* (C: 20.4 ± 6.8; F: 28.5 ± 3.1) increased also after therapy discontinuation (Fig. 6B). Indeed, the families *Bacteroidaceae* (C: 2.1 ± 0.4; F: 1.8 ± 0.6), Prevotellaceae (C: 2.1 ± 1.8; F: 3.1 ± 0.7), S24-7 (C: 24.9 ± 1.1; F: 22.2 ± 4.4) were the most abundant, followed by *Lactobacillaceae* (C: 14.0 ± 8.2; F: 16.8 ± 5.4), *Lachnospiraceae* (C: 13.1 ± 1.9; F: 11.1 ± 3.3), *Ruminococcacea* (C: 23.3 ± 4.3; F: 12.9 ± 5.5) (Fig. 6B). At genus level, *Bacteroides* (C: 2.1 ± 0.7; F: 1.8 ± 0.6), *Prevotella* (C: 2.9 ± 1.8; F: 3.1 ± 1.6), *S24-7* (C: 24.9 ± 5.1; F: 22.6 ± 7.7), *Lactobacillus* (C: 14.0 ± 8.2; F: 18.8 ± 4.7), *Oscillospira* (C: 9.1 ± 1.1; F: 4.3 ± 3.6), *Lachnospira* (C: 4.1 ± 0.9; F: 2.3 ± 0.4), *Ruminococcus* (C: 5.0 ± 1.3; F: 4.9 ± 1.6) and *Coprococcus* (C: 4.4 ± 1.8; F: 3.8 ± 1.7) were the most representative bacteria (Fig. 6B). *Oscillospira* genus, belonging to

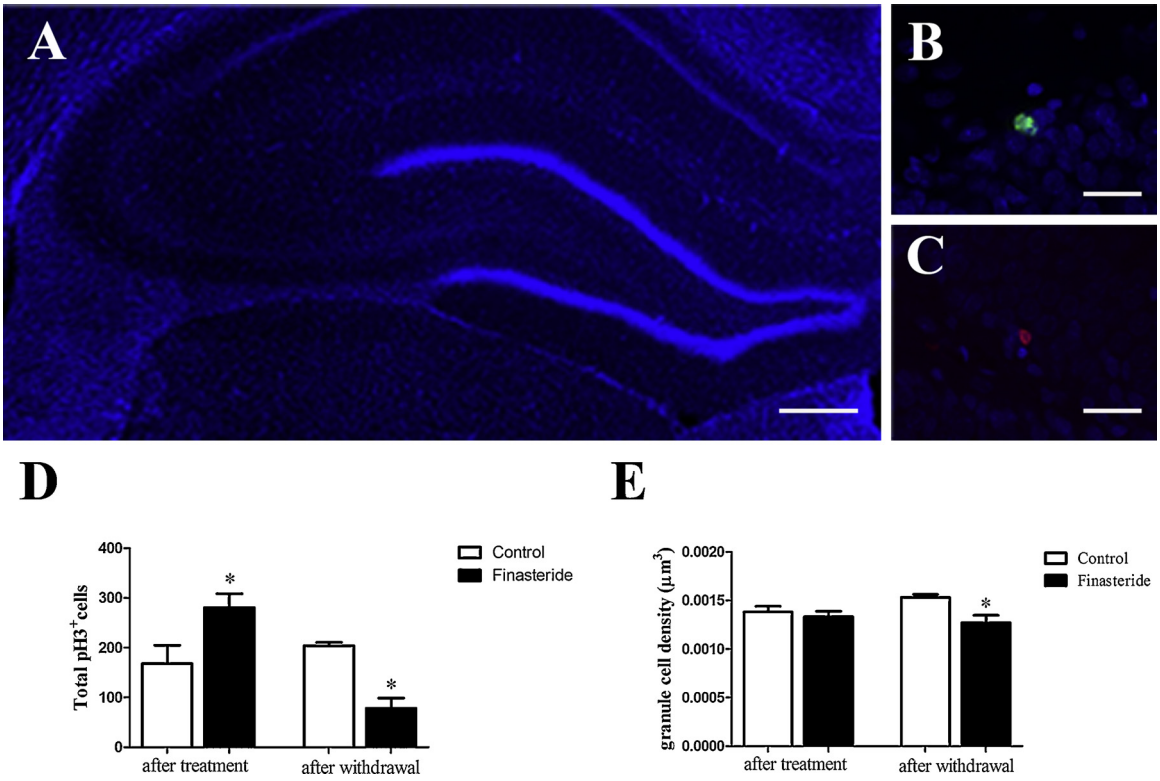

Fig. 2. Effects of finasteride on adult hippocampal neurogenesis. A) Representative DAPI staining of the dentate gyrus. Scale bar, 400 µm. B) Example of a pH3 immunoreactive cell (green) by confocal microscopy in the subgranular layer of the dentate gyrus. The section was counterstained with DAPI. Scale bar, 25 µm. C) Example of a BrdU immunoreactive cell (red) by confocal microscopy in the subgranular layer of the dentate gyrus. The section was counterstained with DAPI. Scale bar, 25 µm. D) Number of pH3 immunoreactive cells in the subgranular layer of the dentate gyrus in animals treated with vehicle (control) or finasteride at the end of the treatment and at one month after withdrawal. Data are expressed as mean ± SEM, n = 4 for each experimental group. *, significant differences, $p < 0.05$, versus control value. Mann-Whitney non-parametric test. E) Density of granule cells in the granular layer of the dentate gyrus in animals treated with vehicle (control) or finasteride at the end of the treatment and at one month after withdrawal. Data are represented as mean ± SEM, n = 4 for each experimental group. *, significant difference, $p < 0.05$, versus the control value at the end of the withdrawal period. Mann-Whitney non-parametric test (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Ruminococcaceae family, and *Lachnospira* were significantly depleted in finasteride group ($p = 0.04$, $p = 0.04$ and $p = 0.03$, respectively, Fig. 6B).

4. Discussion

The findings of the present study indicate that finasteride treatment has persistent effects on depressive-like behavior in young adult male rats. To assess depressive-like behavior we used the Porsolt forced swim test (Castagne et al., 2011). Immobility floating behavior in this test is decreased by antidepressant drugs and is considered a manifestation of helplessness (Castagne et al., 2011). In the forced swim test (as the tail suspension test, the foot shock or any other learned helplessness paradigms) the measurable depression-like state or learned helplessness is inextricably linked to the stress induced to measure it. Different points of view have been reported discussing whether the test is best for measuring either stress-coping or depression (Commons et al., 2017; Cryan and Holmes, 2005; de Kloet and Molendijk, 2016), while some others have reported that long-term administration of Porsolt-efficient antidepressant drugs impairs the stress-coping strategies in mice (Baek et al., 2015), but this debate is beyond our scope, because depression may be considered a stress-coping disorder in many dimensions (Yancura and Aldwin, 2008), stress can induce depression (Larrieu and Sandi, 2018), and depression can impair stress-coping abilities (Michl et al., 2013). The fact is that some of the neural circuits involved in performance of the task, the neurotransmitter levels affected, and the mechanisms underlying the recovery of the stress/depression state by antidepressant drugs, notably mimic that of a human depression (Post and Warden, 2018). In our study, finasteride treatment increased immobility time in the forced swim test compared to vehicle treated animals. This effect of finasteride was not detected at the end of the treatment, but it was observed one month after the end of the treatment. This finding is in agreement with the observation of a persistent depressive symptomatology in a subset of PFS young male patients that received finasteride for the treatment of alopecia (Giatti et al., 2018).

Changes in hippocampal neurogenesis in rodents have been associated with modifications in depressive-like behaviors (Santarelli et al., 2003). Thus, decreased neurogenesis in the dentate gyrus is linked to increased immobility in the forced swim test (Snyder et al., 2011). In agreement, the number of pH3 immunoreactive cells was significantly decreased in the subgranular zone of the dentate gyrus one month after finasteride withdrawal compared to animals injected with vehicle. This finding is compatible with a decrease in neurogenesis in the animals that show depressive-like behavior one month after the end of finasteride treatment and is in agreement with a significant decrease in the granule cell density in the granular layer of the dentate gyrus in these animals. Interestingly, at the end of finasteride treatment, the number of pH3 immunoreactive cells was increased, compared to vehicle injected animals. This suggests that finasteride may first increase neurogenesis, but as a long-term effect, decreases neurogenesis. Our data suggest that the after-withdrawal lower cell survival, both in the mature granule cell population and the newborn cells, might also be present from the very early stage of the treatment, but in this case, compensatory mechanisms seem to be still possible as suggested by the increase in pH3+ cells in the subgranular zone just after one month of continuous treatment. These compensatory mechanisms after one month of

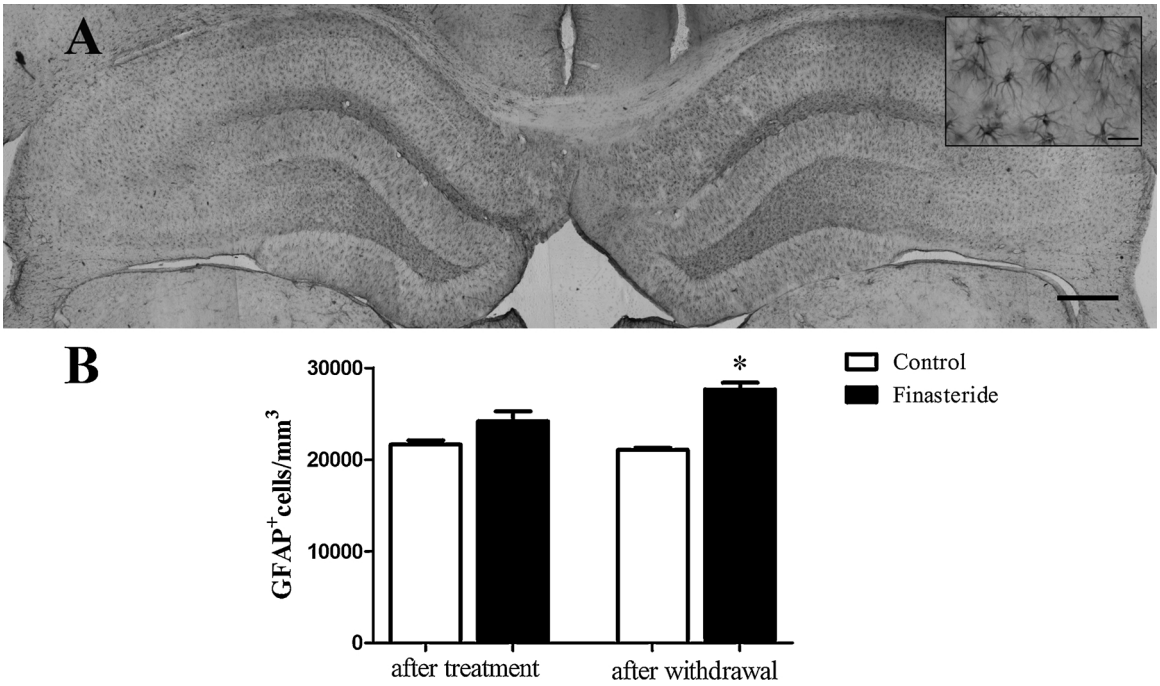

Fig. 3. Effect of finasteride on the number of GFAP immunoreactive astrocytes in the hilus of the dentate gyrus. A) Representative image of the whole dentate gyrus showing immunoreactivity for GFAP. Scale bar 600 μm. The insert shows GFAP immunoreactive astrocytes in the hilus at higher magnification. Scale bar 20 μm. B) Number of GFAP immunoreactive cells/mm^3 in the hilus of the dentate gyrus in animals treated with vehicle (control) or finasteride at the end of the treatment and at one month after withdrawal. Data are expressed as mean ± SEM, n = 4 for each experimental group. *, significant difference, $p < 0.05$, versus control value at the end of the withdrawal period. Mann-Whitney non-parametric test.

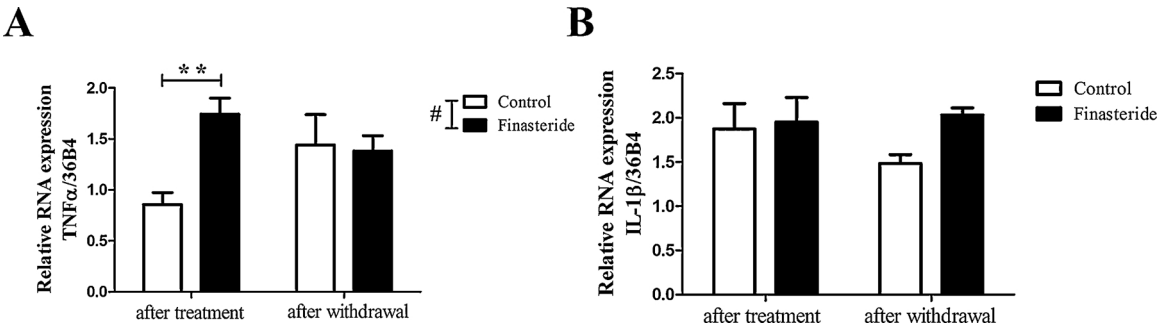

Fig. 4. Effect of finasteride on the mRNA levels of TNFα and IL-1β in the hippocampus. A) TNFα mRNA levels in animals treated with vehicle (control) or finasteride at the end of the treatment and at one month after withdrawal. Data are expressed as mean ± SEM, n = 6 for each experimental group. The effect of treatment, time and the interaction treatment by time were analyzed using two-way ANOVA (significance: # $p < 0.05$) followed by Bonferroni post hoc test (significance: ** $p < 0.01$). B) IL-1β mRNA levels in animals treated with vehicle (control) or finasteride at the end of the treatment and at one month after withdrawal. Data are expressed as mean ± SEM, n = 6 for each experimental group.

treatment are also supported by the fact that 24 h survival of BrdU is not significantly increased when cell proliferation (pH3) is increased. On the contrary, one month after withdrawal of treatment, the cell survival is dramatically reduced in the granular cell layer (as the cell density has been reduced a 17% in the granular cell layer highly packed population, consisting of around 1 million cells per hemisphere in control animals), and this sole effect can well lead to a relevant depletion of the neurogenic pool (as we found in our data), if the mentioned compensatory process has been working for a long time.

Our findings are in line with previous observations, obtained in a different animal model and with a different experimental schedule, in which finasteride treatment induced a decrease of hippocampal neurogenesis (Romer et al., 2010). Interestingly, depressed patients show altered hippocampal morphology (i.e., reduced volume, reduced dendritic complexity and neuronal soma size) as well as reduced hippocampal neurogenesis (Stockmeier et al., 2004).

In both humans and animal models, depressive behaviors are associated with increased neuroinflammation and reactive gliosis (Yirmiya et al., 2015). Microglia and astrocytes are the main mediators of the inflammatory response. Reactive astrocytes show metabolic changes and are less efficient in the supply of lactate to neurons, which is necessary for a proper synaptic function (Steele and Robinson, 2012). Reactive microglia are also less efficient in the maintenance of synaptic function (Tay et al., 2017). Interestingly, finasteride treatment resulted in a significant increase in the number of GFAP immunoreactive astrocytes in the hilus of the dentate gyrus by one month after the end of the treatment compared to vehicle injected animals. This observation, is in principle compatible with an effect of finasteride on astrogliosis. However, finasteride treatment did not significantly change the number of Iba-1 immunoreactive cells in the hilus, suggesting that finasteride did not induce a full gliotic response involving both astrocytes and microglia, but specifically affects the activation of astrocytes. In this regard it is important to note that one month after finasteride withdrawal, there is a significant increase in the levels of DHP in the male

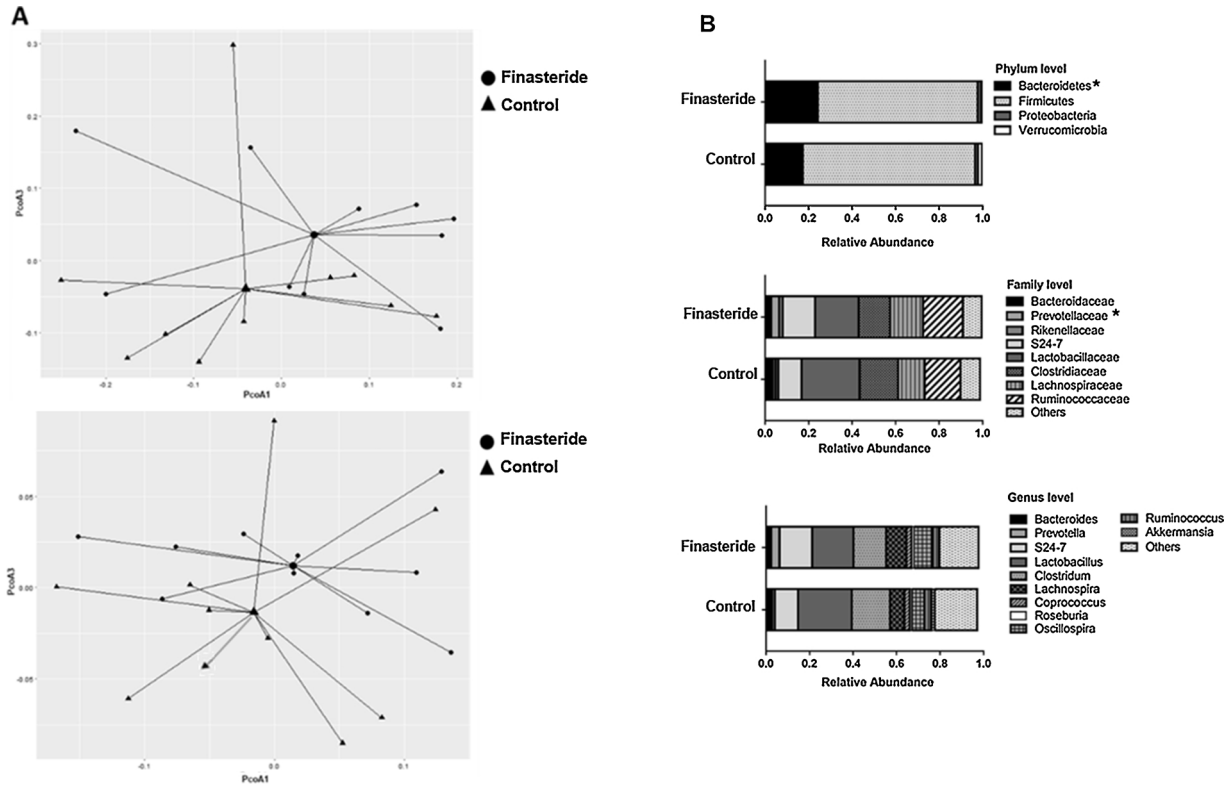

Fig. 5. Gut microbiota modulation at the end of finasteride treatment. A) Principal Coordinate Analysis (PCoA, beta-diversity) plot according to unweighted (up panel) and weighted (down panel) Unifrac distances. B) Relative abundances in gut microbiota. Histograms of relative abundances at phylum, family, and genus. Statistical significant values ($* p < 0.05$) are reported highlighting the group with greater abundance.

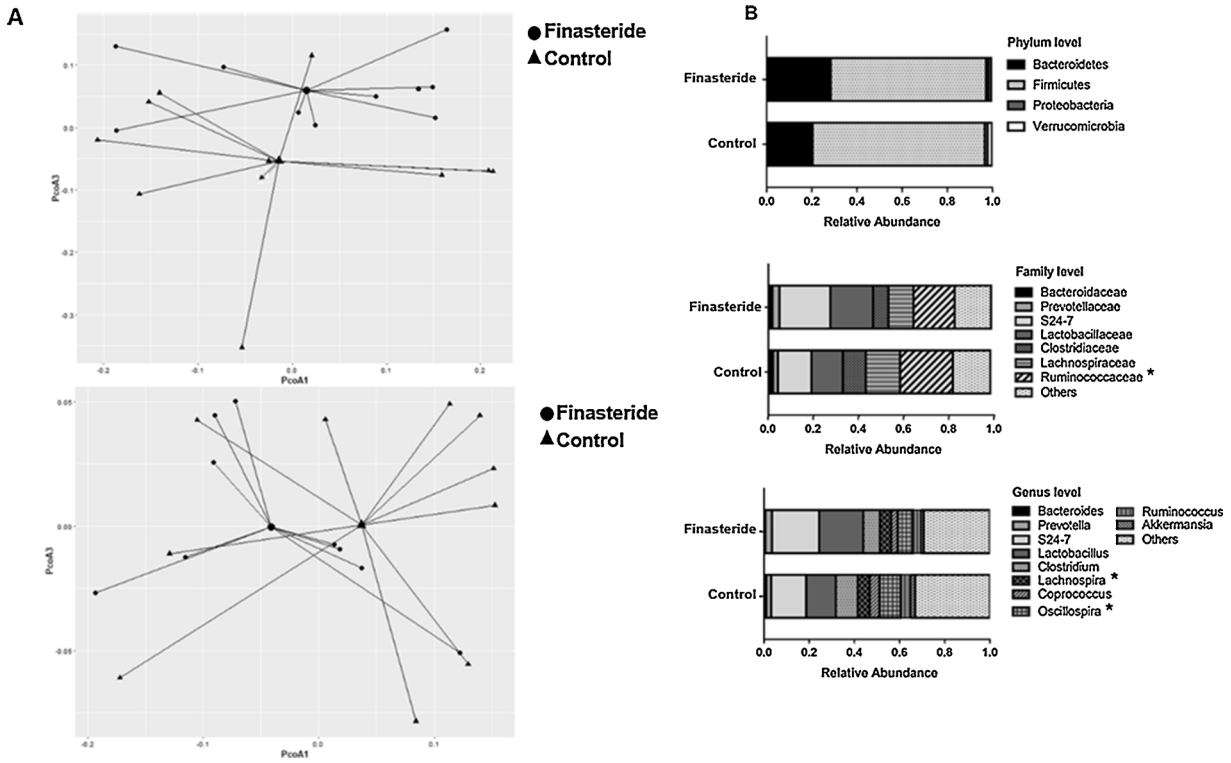

Fig. 6. Gut microbiota composition 1 month after finasteride withdrawal. A) Principal Coordinate Analysis (PCoA, beta-diversity) plot according to unweighted (up panel) and weighted (down panel) Unifrac distances. B) Relative abundances in gut microbiota. Histograms of relative abundances at phylum, family, and genus. Statistical significant values ($* p < 0.05$) are reported highlighting the group with greater abundance.

rat hippocampus compared to control animals (Giatti et al., 2016). Interestingly, DHP is known to increase GFAP gene expression in astrocytes (Giatti et al., 2012). Therefore, the increase in the number of GFAP immunoreactive astrocytes one month after finasteride treatment may represent an increase in GFAP expression by the elevated levels of DHP. Since GFAP plays an essential role in the regulation of astrocyte morphology and is involved in astrocyte-neuron communication (Sofroniew and Vinters, 2010), changes in GFAP levels one month after finasteride withdrawal may affect hippocampal function and contribute to depressive-like behavior.

Reactive gliosis is associated with increased levels of proinflammatory molecules in the brain (Wang et al., 2018). In agreement with this, the effect of finasteride on the number of GFAP immunoreactive astrocytes was associated with changes in the mRNA levels of the proinflammatory cytokines. Indeed, finasteride increased the levels of TNF-α at the end of the treatment compared to vehicle injected animals. TNF-α has been shown to disrupt the blood brain barrier and this results in prolonged depressive-like behavior in mice (Cheng et al., 2018). Thus, the increase in the levels of TNF-α by finasteride may cause long term effects in depressive-like behavior, such as the increased immobility observed in animals one month after finasteride withdrawal. On the contrary, by two-way ANOVA analysis, the mRNA levels of IL-1β in the hippocampus were not significantly different. However, it is important to note that, analyzing these data by Student's t-test, a significant increase of the gene expression of this cytokine was observed after withdrawal ($p < 0.01$, $t = 4.351$, df: 10).

Interestingly, the expression of both cytokines is increased in plasma of patients with depression and in the brain of depression animal models (Cheng et al., 2018; Wang et al., 2018). Last but not least, it is relevant to take into account that performance of the forced swim test induced stress/depression on all animals before measuring the brain-related parameter described here. As the background of the work stems for the data collected after treatment-induced depression in originally healthy patients, the main conclusions of our manuscript are still pertinent. Certainly, further investigation is needed to extend the effect of finasteride treatment and withdrawal described here to non-stressed subjects.

Data here presented also show for the first time that a subchronic treatment with finasteride was able to affect gut microbiota of male rats. Indeed, an increase in *Bacteroidetes* phylum as well as in *Prevotellaceae* family was observed. Interestingly, gut microbiota is also affected at the finasteride withdrawal, even if in a different way. Indeed, a decrease in *Ruminococcaceae* family as well as *Oscillospira* and *Lachnospira* genus was detected. These observations suggest that changes in plasma neuroactive steroid levels caused by 5α-R inhibition in male rats (Giatti et al., 2016) are responsible for the gut microbiota modulation here observed. Indeed, at least for peripheral steroid environment (i.e., steroid hormones coming from peripheral glands) a relationship with gut microbiota has been already proposed (Tetel et al., 2018). Gonadectomy and hormone replacement have a clear effect on gut bacteria in rodents (Harada et al., 2016; Moreno-Indias et al., 2016; Org et al., 2016). For instance, *Ruminococcaceae* are significantly affected by orchidectomy in mice (Org et al., 2016). On the other hand, in the experimental model used in the present study, steroid levels are not only altered in plasma, but also in different brain regions (Giatti et al., 2016). On the basis of the existence of a gut microbiota-brain axis (Mayer, 2011; Sharon et al., 2016), it cannot be excluded that modifications in neural function, as a consequence of the changes in brain levels of neuroactive steroids, may in turn alter gut microbiota control. Although 5α-R activity has not been evaluated so far in gut microbiota, some observations seem to indicate that intestinal epithelial cells are able to synthesize glucocorticoids (Cima et al., 2004) and that some microbial species, such as *Clostridium scindens* have the potentiality to convert glucocorticoids into androgens (Ridlon et al., 2013). Therefore, an alternative possibility is a direct action of finasteride on gut microbiota.

In addition, it is also interesting to note that changes in gut microbiota we here observed are very similar to what observed in patients with major depressive disorder (Jiang et al., 2015) as well as in animal models of depression. For instance, rats with depressive-like behavior show an increase in *Bacteroidetes* (Yu et al., 2017). Interestingly, in agreement with what reported in other experimental models we also here observed that gut microbiota changes are related with changes in adult hippocampal neurogenesis, glial reactivity and neuroinflammation (Mohle et al., 2016; Rea et al., 2016). Moreover, as previously reported (Giatti et al., 2016), finasteride withdrawal induced a decrease in the gene expression of the alpha4 and beta3 subunits of the GABA-A receptor in the cerebral cortex. This effect was associated with the decrease in the brain levels of allopregnanolone and isopregnanolone, two DHP metabolites that are able to modulate the activity of this neurotransmitter receptor (Giatti et al., 2016). Both GABA and the above mentioned neuroactive steroids are also implicated in the pathogenesis of depression (Melcangi et al., 2016; Schule et al., 2014). Interestingly, some members of human microbiota (i.e. *Bifidobacterium* spp. and *Lactobacillus* spp.) encode for genes involved in GABA production, suggesting a microbial participation in the production of this neurotransmitter within the gut (Barrett et al., 2012). Indeed, administration of *Lactobacillus rhamnosus* to mice is able to counteract depression-related behavior by directly increasing GABA levels and, indirectly through the vague nerve, by modulating GABAergic neurotransmission (Bravo et al., 2011). Other microbial metabolites, mainly butyrate, have been demonstrated to play a role in ameliorating depression symptoms (Gundersen and Blendy, 2009). Therefore, these findings are in agreement with the concept of a bidirectional communication between the gut microbiota and the nervous system, suggesting that neuroactive steroids and GABA are involved in this communication.

5. Conclusion

In summary, our findings indicate that finasteride treatment causes several alterations in the hippocampus that are detected at the end of the treatment, such as increased proliferation in the subgranular zone of the dentate gyrus, increased number of astrocytes in the hilus and increased mRNA levels of TNF-α. In addition, other changes are detected by one month after finasteride withdrawal, including decreased proliferation in the subgranular zone, increased astrogliosis in the hilus and a possible (i.e., only detected by Student's t-test) increased of mRNA levels of IL-1β in the hippocampus. These latter changes coincide with the apparition of depressive-like behavior, suggesting that long term effects of finasteride treatment on neurogenesis and neuroinflammation may participate in the enduring effects of the drug on depressive-like behavior, which are detected even one month after stopping the administration of the drug. It should be noted that important changes in the levels of neuroactive steroids are detected in the hippocampus and other brain regions by one month after finasteride withdrawal (Giatti et al., 2016). Since neuroactive steroids regulate neurogenesis, gliosis and neuroinflammation (Galea, 2008; Giatti et al., 2012; Melcangi et al., 2016) and since PFS patients also show changes in neuroactive steroid levels (Caruso et al., 2015; Melcangi et al., 2013, 2017), the effect of finasteride on depression, neuroactive steroid levels, neurogenesis and neuroinflammation may be interrelated events. In addition, the changes here observed at the end of treatment and at withdrawal on gut microbiota may depict further possible signals involved in the so call *gut microbiota-brain axis*.

Contributors

Silvia Diviccaro performed the treatment of animals, behavioral analysis, immunohistochemistry, morphometric analysis, granule cell density analysis.

Silvia Giatti performed assessment of cytokines by Real time PCR

Francesca Borgo, Elisa Borghi and Matteo Barcella responsible for gut microbiota analysis

Silvia Diviccaro, Francesca Borgo, José Luis Trejo, Luis Miguel Garcia-Segura and Roberto Cosimo Melcangi were responsible for study design and wrote the manuscript.

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Conflicts of Interest

None.

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