UNIVERSITÀ DEGLI STUDI DI MILANO

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Effect of finasteride treatment in male rats: depressive-like behavior and related parameters

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Index

Abstract

Neuroactive steroids are important physiological modulators of the nervous system as well as protective agents in different psychiatric and neurodegenerative diseases. This group of molecules includes hormonal steroids released by peripheral steroidogenic tissues and neurosteroids directly synthetized in nervous system. One key process regulating the synthesis of these molecules is the enzyme 5α-reductase (5α-R). This enzyme converts progesterone (PROG) and testosterone (T) to their 5α-reduced metabolites, dihydroprogesterone (DHP) and dihydrotestosterone (DHT), respectively. These metabolites are then further converted into other neuroactive steroids, such as tetrahydroprogesterone (THP) and isopregnanolone (ISOPREG) in the case of DHP and 5α-androstane-3α, 17β-diol (3α-diol) and 5α-androstane-3β, 17β-diol (3β-diol) in the case of DHT. In agreement, with the protective role exerted by neuroactive steroids their levels are affected in neurodegenerative as well as psychiatric disorders. In particular, 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 5α-R. Thus, these patients are affected by the so-called post-finasteride syndrome (PFS). Interestingly, suspension of finasteride treatment did not only lead, as expected, to an alteration of 5α-reduced metabolites of PROG and T but also to a global alteration of neuroactive steroid levels. Moreover, recent observations performed in male rats, show that this persistent alteration in neuroactive steroid levels did not only occur in plasma and CSF but also in the brain.

On this basis, in the present PhD project, we have ascertained whether subchronic finasteride treatment (i.e. 20 days) and one month of its withdrawal may induce depressive-like behavior in this animal model. In addition, the effect of finasteride on other depressive-related parameters such as neurogenesis, gliosis, neuroinflammation/inflammation and gut microbiota have been analysed.

At the end of finasteride treatment, we observed: 1) increased proliferation in the subgranular zone of the dentate gyrus; 2) increased number of microglia with reactive phenotype in the hilus and 3) increased mRNA levels of TNF-α. One month after finasteride withdrawal, we reported: 1) decreased proliferation in the subgranular zone; 2) decreased granule cell density in the granule cell layer; 3) increased astrogliosis in the hilus and 4) a possible (i.e., only detected by Student's t-test) increased of mRNA levels of IL-1β in the hippocampus. Moreover, these latter changes coincide with the onset of depressive-like behavior, suggesting that long-term effects of finasteride treatment on neurogenesis and neuroinflammation may participate in the lasting effects of the drug on depressive-like behavior, which are detected even one month after discontinuation of the drug.

In addition, peripheral inflammation assessment revealed, after drug treatment, a significant decrease in plasma levels of IL-1β in finasteride-treated vs control animals. On the other hand, in agreement with literature and with the depressive-like behavior, a significant increase in plasma levels of IL-1β in finasteride-treated vs control animals at withdrawal period was observed. These observations seem to support a role of peripheral inflammation in addition to what we reported on neuroinflammation.

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.

It should be noted that important changes in the levels of neuroactive steroids such as PROG and DHP are detected in the hippocampus by one month after finasteride withdrawal. Since neuroactive steroids regulate neurogenesis, gliosis and neuroinflammation, and since PFS patients also show changes in neuroactive steroid levels, the effect of finasteride on depression, neuroactive steroid levels, neurogenesis and neuroinflammation/inflammation 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.

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

Abbreviations

36B4: ribosomal protein 36B4
3α-diol: 5α-androstane-3α,17β-diol
3β-diol: 5α-androstane-3β,17β-diol
3β-HSD: 3β-hydroxysteroid dehydrogenase
3α-HSOR: 3α-hydroxysteroid oxidoreductase
3β -HSOR: 3β -hydroxysteroid oxidoreductase
5α-R: 5α-reductase
17β-HSD: 17β-hydroxysteroid dehydrogenase
17β-E: 17β-Estradiol
ARs: androgen receptors
ARO: aromatase
CNS: central nervous system
CSF: cerebrospinal fluid
DHDOC: dihydrodeoxycorticosterone
DHEA: dehydroepiandrosterone
DHP: dihydroprogesterone
DHT: dihydrotestosterone
ERs: estrogen receptors
GABA-A/B: gamma aminobutyric acid-A/B
HSL: hormone-sensitive lipase
IL: interleukin
IMM: inner mitochondrial membrane
i.p.: intra-peritoneal
ISOPREG: isopregnanolone
MDD: major depressive disorder
OMM: outer mitochondrial membrane
P450scc or CYP11A1: cytochrome P450 side chain cleavage
PNS: peripheral nervous system
PRs: progesterone receptors
PREG: pregnenolone
PROG: progesterone
StAR: steroidogenic acute regulatory protein
T: testosterone
THP: tetrahydroprogesterone
TNF-α: tumor necrosis α
TSPO: 18 kDa translocator protein
VDAC: voltage-dependent anion channel

1. INTRODUCTION

1.1. Neuroactive steroids: steroid hormones and neurosteroids

Steroid hormones are lipid molecules synthesized in peripheral glands such as gonads or adrenal cortex. Steroids hormones interact with nuclear receptors as well as with membrane receptors (Brinton *et al*, 2008; Melcangi *et al*, 2008a). The classical steroid receptors (i.e. nuclear receptors) natively localized in the cytoplasm, are activated by binding with the hormone, and then move into the nucleus where they perform their regulatory action on DNA. Progesteron (Blaustein, 2003), androgen (Cato and Peterziel, 1998), estrogen (Shupnik, 2002), glucocorticoid and mineralocorticoid receptors (McEwan et al, 1997) may be included in this category. The activity of these receptors leads to trigger the medium- and long-term steroid hormones effects such as the sexual differentiation or the secretion of hypophyseal hormones (see review Handa and Weiser, 2014). Furthermore, additional observations have assessed that steroids are also able to trigger short-term effects, i.e. effects that take place in short time as seconds or minutes. This molecular mechanism suggests the existence of a new receptors class, the so-called non-classical steroid receptors, which are located within the membrane and, seem to mainly trigger short-term effects. GABA type A and type B receptors (GABA-A receptor, GABA-B receptor), serotonin type 3 (5-HT3), kainate receptor, alpha-amino-3hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-Methyl-D-aspartate (NMDA) and an atypical intracellular receptor like the sigma 1 may be included in this category (Melcangi et al, 2005).

Steroid hormones by their lipophilic structure, may diffuse across the cell membranes and then bind their receptors in the tissues. Their structure consists of a tetracyclic system of carbon atoms, called cyclopentanoperidrofenantrene or sterane. It consists of four cast rings, which of them three esameric- and one pentameric-carbon cycle, i.e. the typical basic structure of cholesterol. Steroid hormones have then various substituents that determine the specificity of action on certain receptors. These molecules may be classified into different groups: androgens (testosterone and derivatives), estrogens (estradiol), progestogens (progesterone and derivatives) and corticosteroids, which in turn include glucocorticoids (cortisol) and mineralcorticoids (aldosterone) (Figure 1).

Several data in literature have demonstrated that these molecules exert their activity on the central (CNS) and peripheral nervous system (PNS), controlling important physiological processes such as behavior, memory, the brain sexual differentiation and reproduction (Fink *et al*, 1991; McEwen, 1981, 1994). The mechanism by which these molecules exert their effects in the nervous system is usually considered the classical endocrine mechanisms. As concern this mechanism, steroid

hormones are produced by endocrine organs such as gonads and the adrenals glands, secreted into the blood, and then they regulate CNS functions, after crossing the blood-brain barrier.

However, steroid hormones released by peripheral steroidogenic tissues, are not the only endocrine molecules acting on the nervous system. Indeed, it was demonstrated that the nervous system may synthesize steroid molecules (Corpechot *et al*, 1981; Giatti *et al*, 2015c). Indeed, significant amount of dehydroepiandrosterone (DHEA), PREG and their sulfate esters (PREGS and DHEAS) in the mammalian brain was demonstrated after 15 days of adrenalectomy and orchidectomy (Corpechot *et al*, 1983). Baulieu and co-workers, called these molecules "neurosteroids". Thus, steroids directly synthesized by neurons and glial cells, which have the ability to regulate the nervous system function, with an autocrine/paracrine mechanism of action (Baulieu, 1998). However, several further investigations, using different animal models, have been required before consolidation of this new concept (Baulieu, 1999; Compagnone and Mellon, 2000; Mensah-Nyagan *et al*, 1999; Schumacher *et al*, 2003). These investigations have significantly increased the basic knowledge on neurosteroids, leading to an exact definition highlighting new specific identification criteria. Thus, the candidate steroidal molecule, may be only considered as neurosteroid if it persists in the nervous system in a substantial amount, after removal of the peripheral steroidogenic glands such as the adrenals and gonads (Baulieu, 1999). On the other hand, since the nervous system is a target for two different pools of steroids, steroid hormones and neurosteroids, (i.e., one coming from the peripheral glands and the second one originating directly in the nervous system, respectively) in many circumstances, it is difficult to assign which is the responsible of the effects on the nervous system.

Therefore, the investigators of this field use now the term "neuroactive steroids" (Paul and Purdy, 1992) which includes steroid hormones and neurosteroids.

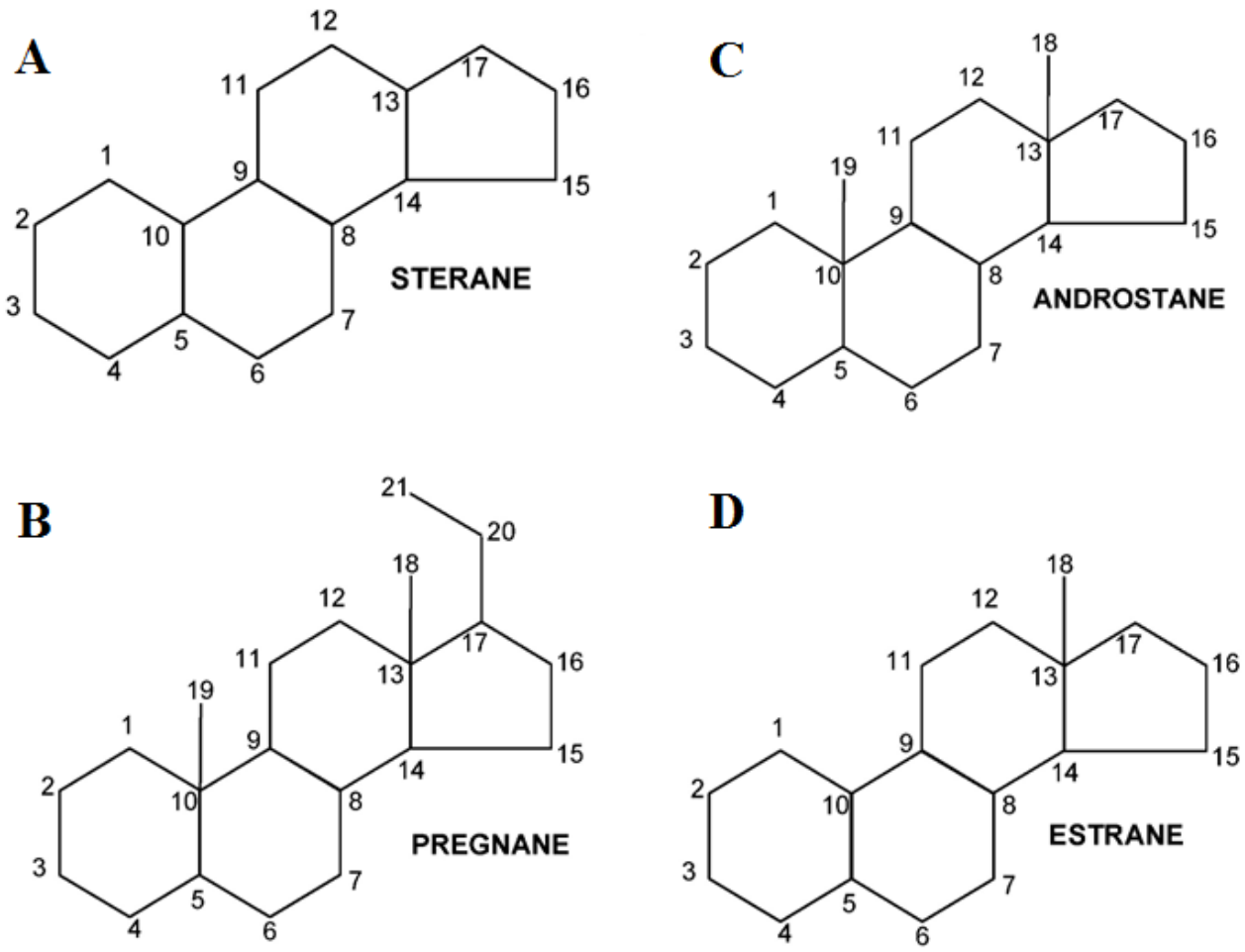

Figure 1. *Chemical structure of steroids. A) Sterane or cyclopentanoperidrofenantrene, basic structure of all steroidal compounds; B) pregnane, the core characterizing all C21 steroids such as progestogens and corticosteroids; C) androstane, characteristic nucleus of C19 steroids such as androgens; D) estrane, characteristic core of C18 steroids such as estrogens. Figure modified by (Mensah-Nyagan et al, 2009).*

It is now clear that neuroactive steroids are important physiological regulators of nervous function. In addition, these molecules are also protective in neurodegenerative and psychiatric disorders (Garcia-Segura *et al*, 2001; Giatti *et al*, 2018b; Giatti *et al*, 2015a; Sousa *et al*, 2000; Wirth, 2011). For these reasons, due to their ability to control not only homeostatic parameters but also to protect the nervous system during crucial pathophysiological process, in the last decades, neuroactive steroids have received a great amount of attention (Charalampopoulos *et al*, 2006; Dubrovsky, 2006; Melcangi and Garcia-Segura, 2006; Melcangi and Panzica, 2006).

1.2. Biosynthesis and metabolism of steroids in the nervous system

The demonstration of the biosynthesis of these steroids comes from evidence that underline the expression and biological activity of the key enzymes of steroidogenesis in nervous cells. These enzymes are involved in the translocation of cholesterol from intracellular stores to the inner mitochondrial membrane, which is the first step of synthesis. This carriage occurs through a molecular complex constituted by the steroidogenic acute regulatory protein (StAR), the translocator protein 18 kDa (TSPO) and the voltage-dependent anion channel protein (VDAC) (Lavaque *et al*, 2006b; Papadopoulos *et al*, 2006a; Papadopoulos and Miller, 2012; Sierra *et al*, 2003). Furthermore, there are numerous key enzymes involved in the steroids synthesis as well as in the conversion of these into neuroactive metabolites. Initially, in the inner mitochondrial membrane, cholesterol is actively converted into pregnenolone (PREG) by the cytochrome P450 side-chain cleavage (P450scc) enzyme (Compagnone *et al*, 1995; Le Goascogne *et al*, 1987) (See figure 2). This is the first, rate-limiting, and hormonally regulated step in the synthesis of these molecules. Thus, it is the expression of CYP11A1 (i.e., P450scc gene) and the respective presence of the protein that makes a cell "steroidogenic" and able to produce steroids *de novo*, as opposed to modifying steroids produced elsewhere, which occurs in many types of cells. Then, PREG diffuses into the cytosol where is further transformed into PROG or DHEA in the endoplasmic reticulum (Figure 2). Indeed, further steroidogenic enzymes, such as cytochrome P450c17 (P450c17), 3β-hydroxysteroid dehydrogenase (3β-HSD), 5α-reductase (5α-R), 3α-hydroxysteroid oxidoreductase (3α-HSOR), 3β-hydroxysteroid oxidoreductase (3β-HSOR), 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase (CYP19) were also identified (Baulieu, 1999; Compagnone *et al*, 2000; Melcangi *et al*, 2008a; Mensah-Nyagan *et al*, 1999; Schumacher *et al*, 2003).

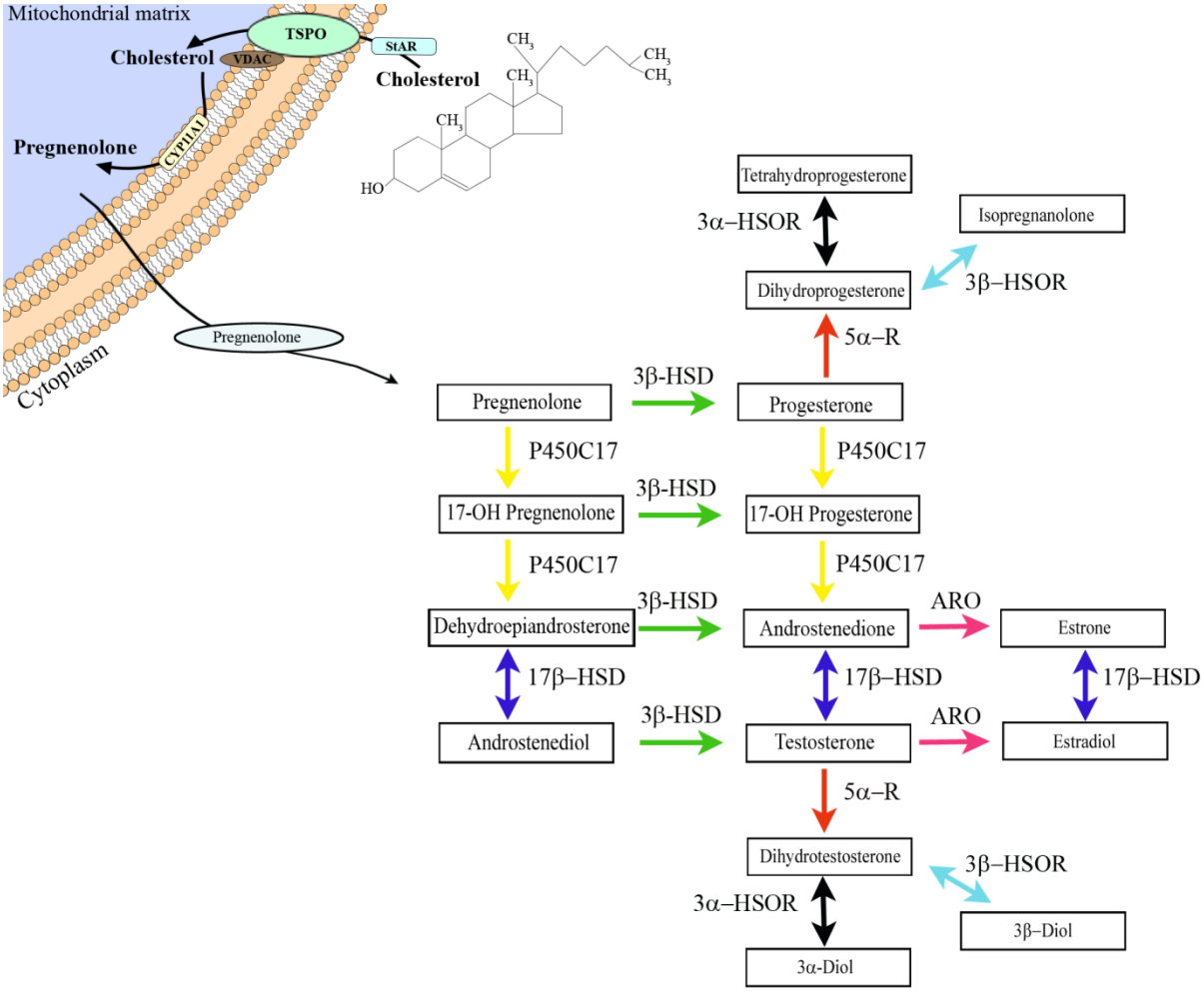

Figure 2. *Schematic representation of steroidogenic pathway. The arrows indicate the irreversible reactions and bidirectional arrows are reversible reactions performed by each enzyme: yellow = P450C17; blue = 17β-HSD; green = 3β-HSD; red = 5α-R; light blue = 3β-HSOR; black = 3α-HSOR; pink = ARO. The first step is the entry of cholesterol from the cytosol to the inner mitochondrial membrane, via the steroidogenic acute regulatory protein (StAR), the 18 kDa translocation protein (TSPO) and voltage-dependent anion channel (VDAC), where the conversion into pregnenolone (PREG) by the P450scc occurs. Successively, PREG diffuses into cytoplasm where is converted into the other metabolites.*

Briefly, PROG and DHEA are molecules that can directly exert their effect on the nervous system but can also undergo subsequent transformations and act through their metabolites. In all CNS cells, DHEA may be converted into androstenediol and then into testosterone (T) or transformed into androstenedione (Melcangi *et al*, 2008a). In neurons and astrocytes, androstenedione, as well as T, may be converted by aromatase P450 (ARO) into estrone and estradiol respectively, but this does not occur in microglia because it does not express the enzyme (Garcia-Segura *et al*, 2003).
Finally, in neurons, as well as in astrocytes, PROG and T may be metabolized by the enzyme complex consisting of 5α-R and 3α-HSOR or 3β-HSOR in dihydroprogesterone (DHP), tetrahydroprogesterone (THP, also known as allopregnanolone) and isopregnanolone in case of PROG, and dihydrotestosterone (DHT), 5α-androstane-3α, 17β-diol (3α-diol) and 5α-androstane-3β, 17β-diol (3β-diol) in case of T (see for review Melcangi *et al*, 2008a).

1.2.1. StAR and TSPO

As mentioned above, the first step is the translocation of cholesterol from the cytoplasm to the mitochondrial membrane. This is a limiting step hormonally mediated by the steroidogenic acute regulatory protein (StAR) (Lavaque *et al*, 2006a; Lavaque *et al*, 2006b).
Previously, it was proposed a StAR isoform of 37 kDa as a precursor of the functional, intra-mitochondrial protein of 30 kDa. However, recent observations indicate that the 37 kDa StAR isoform acts as mature isoform on the outer mitochondrial membrane (OMM). Then, it is rapidly transported into mitochondria where it is cleaved generating a functional 30 kDa intra-mitochondrial StAR protein that, after phosphorylation, acts on the inner mitochondrial membrane (IMM) for the cholesterol transport (Artemenko *et al*, 2001; Manna *et al*, 2016; Miller, 2013). The transport of cholesterol from the outer to the inner mitochondrial membrane is the mechanism that mediates the rate-limiting step in steroid biosynthesis. However, considerable evidence suggest that a dynamic protein complex is involved in this process (Arakane *et al*, 1998; Artemenko *et al*, 2001; Fan *et al*, 2015; Fan *et al*, 2010; Liu *et al*, 2006; Manna *et al*, 2013; Orlando *et al*, 2013; Poderoso *et al*, 2013; Rone *et al*, 2012). Among the proteins of this dynamic complex, the StAR has essentially all of the characteristics to become an acute regulator of steroid biosynthesis in steroidogenic tissues (Arakane *et al*, 1998; Clark *et al*, 1994; Lin *et al*, 1995; Miller, 2007a, b; Miller and Auchus, 2011; Miller and Bose, 2011; Stocco, 2001; Stocco and Clark, 1996). In agreement, the StAR transcriptional and/or translational inhibition, strongly decreases, but not abolishes, steroid synthesis (Clark *et al*, 1997; Manna *et al*, 2009; Stocco, 2001). This suggest that not only StAR, but also other proteins, are involved in the intra-mitochondrial cholesterol transport that regulate the steroidogenesis (Manna *et*

al, 2016). Indeed, several proteins have been identified, even if their specific role have to be further clarified (Bose *et al*, 2008; Papadopoulos *et al*, 2012; Rone *et al*, 2012). Among these, the peripheral benzodiazepine receptor, now called 18 kDa translocator protein (TSPO), has been the first to be identified (Papadopoulos *et al*, 2006b; Papadopoulos *et al*, 2006c) (Figure 2). This protein was initially proposed as an "acute trigger" of steroidogenesis, but further experiments have now cleared that StAR plays that role while TSPO is part of the molecular machinery through which StAR acts on the OMM (Papadopoulos *et al*, 2012). Moreover, it has been demonstrated its involvement in the translocation of cholesterol from the outer to the inner mitochondrial membrane. Data in literature have shown in different neuropathological conditions such as neurodegenerative disorders, gliomas and neuroinflammation induced by neurotoxins that there is an up-regulation of TSPO expression (Papadopoulos *et al*, 2006b). Furthermore, after CNS injury, the expression of TSPO is principally restricted to microglia and astrocytes (Kassiou *et al*, 2005). StAR may also interact with the voltage-dependent anion channel (VDAC) at the mitochondria-associated endoplasmic reticulum membrane (MAM) before to its translocation to the mitochondrial matrix. VDAC seems also to mediate hormone-trigger cholesterol mobilization to the mitochondrial matrix (Prasad *et al*, 2015). Recently, it was demonstrated that StAR expression is also regulated by hormone sensitive lipase (HSL), a neutral cholesteryl ester hydrolase, which converts cholesteryl esters into free cholesterol for steroid hormone production. In particular, deficiency of HSL affects StAR and steroid levels, demonstrating that HSL plays a crucial role in regulation the steroidogenic response. In agreement, the physical interaction between HSL and StAR, leads to an enhancement of the hydrolytic HSL activity that facilitates the trafficking of intracellular cholesterol from lipid droplets into the mitochondria (Shen *et al*, 2003). Furthermore, the inhibition of HSL activity which blocks the release of cholesterol from lipids droplets, decreases StAR expression and steroid biosynthesis (Soccio *et al*, 2002; Strauss *et al*, 2003; Watari *et al*, 1997; Zhang *et al*, 2002).

StAR seems to be broadly distributed throughout the brain, although different expression levels have been detected among different brain areas. For example, StAR expression appears to be restricted to very specific neuronal and astroglial populations in each brain area, although it is mostly expressed by neurons (Sierra *et al*, 2003). Moreover, it has been demonstrated in the hippocampus that the StAR mRNA level, was approximately 1/100 of that in rat testis (Shibuya *et al*, 2003). The tissue-specific expression of StAR may be explained by its transcriptional regulation. Since StAR mRNA levels are increased rapidly in response to the cellular signals that also drive StAR protein expression and steroidogenesis, it was presumed that StAR transcription and translation are tightly coupled in response to a single signaling event (Manna *et al*, 2009; Manna *et al*, 2007; Manna and Stocco, 2008; Nieschlag *et al*, 2004).

1.2.2. P450scc

As mentioned above, cholesterol, after the translocation from OMM into IMM, is actively converted into PREG (Figure 2) by a mitochondrial enzyme namely cytochrome P450 side chain cleavage (P450scc) encoded by CYP11A1 gene. This first process, involves three chemical reactions, the 22-hydroxylation of cholesterol, 20-hydroxylation of 22(R)-hydroxycholesterol, and oxidative scission of the C20-22 bond of 20(R),22(R)-dihydroxycholesterol (the side-chain cleavage event), to produce PREG (Hall, 1986; Tuckey and Cameron, 1993). On the other hand, soluble hydroxysterols such as 22(R)-hydroxycholesterol may quickly enter the mitochondrion, without the action of StAR and its machinery. Catalysis performed by P450scc is slow, with a net turnover of approximately 6 – 20 cholesterol molecules per molecule of P450scc, every second.

Moreover, the transcription of CYP11A gene is regulated by tissue-specific and hormonally responsive factors, and it may be induced by second messenger system as well as protein kinase A and C acting through different CYP11A1 promoter sequences (Moore *et al*, 1990). In adrenals and gonads, the transcription of P450scc and other steroidogenic enzymes requires the action of steroidogenic factor 1 (SF1) (Schimmer and White, 2010). Conversely, placenta expression of P450scc is constitutive, SF1-independent (Henderson *et al*, 2007; Schimmer *et al*, 2010).

The presence of P450scc in the rat brain white matter was demonstrated by immunohistochemistry (Le Goascogne *et al*, 1987). Moreover, the conversion of cholesterol into PREG was demonstrated by chemical assay in primary culture of rat glial cells (Jung-Testas *et al*, 1989). More recently, Shibuya et co-workers demonstrated that the relative levels of P450scc in the hippocampus were approximately 1/500 than in the rat testis (Shibuya *et al*, 2003).

1.2.3. P450c17

PREG is converted into DHEA by the enzymatic complex P450c17 (Figure 2), a two-step reaction that converts PREG before into 17-hydroxy-PREG and then in DHEA. This enzyme is also responsible for the conversion of PROG into 17-hydroxy-PROG and androstenedione.

In early studies, the production of DHEA in the rat brain was suggested. Indeed, the expression of mRNA encoding P450c17 was shown in cerebellum and brain stem of adult rats (Kohchi *et al*, 1998; Stromstedt and Waterman, 1995). Subsequently, expression and enzymatic activity of P450c17 in adult hippocampal neurons were demonstrated (Hojo *et al*, 2004). Moreover, the first cellular

mapping of an active P450c17 in adult rodent spinal cord suggested that endogenous DHEA synthetized in spinal neural networks may control various spinally-mediated activities (Kibaly *et al*, 2005).

1.2.4. 17β-HSD

In all cells of CNS, 17β-HSD converts DHEA into androstenediol (Jellinck *et al*, 2007) and androstenedione into T (Figure 2). In addition, it is able to convert estrone into estradiol (Jellinck *et al*, 2006; Jellinck *et al*, 2007). In humans, 17β-HSD is present in five isoforms which are expressed in several areas of body (Martel *et al*, 1992). Furthermore, several isoforms of 17β-HSD were also detected in various rodent tissues (Normand *et al*, 1995). For example, in the rat brain, the immunoreactivity for 17β-HSD is widely distributed in hippocampus, hypothalamus, cerebral cortex and thalamus (Mensah-Nyagan *et al*, 1999).

1.2.5 3β-HSD

The conversion of delta-3β-hydroxysteroids (PREG, 17OH-PREG, DHEA) into delta-3-ketosteroids (PROG, 17OH-PROG and androstenedione) is performed by the enzyme 3β-HSD (Figure 2). In human, this enzyme exists in two isoforms: 3β-HSD type 1, which is basically expressed in the placenta and 3β-HSD type 2, which is mostly expressed in the gonads and adrenal glands (Luu The *et al*, 1989; Rheaume *et al*, 1991). In addition, through molecular cloning of the cDNA, six types of 3β-HSD were characterized in mice (Simard *et al*, 1996) and four types in rats (Zhao *et al*, 1991). The observations obtained in homogenates of rat amygdala and septum, demonstrating PREG conversion into PROG were the first result suggesting the existence of 3β-HSD in the CNS (Weidenfeld *et al*, 1980). In addition, the biological activity of 3β-HSD has also been identified in primary cultures of rodent oligodendrocytes and neurons (Jung-Testas *et al*, 1989). The expression of 3β-HSD protein and/or mRNA has been detected in various brain regions of several classes of vertebrates including fish (Mathieu *et al*, 2001; Sakamoto *et al*, 2001), birds (Ukena *et al*, 1999), amphibians (Mensah-Nyagan *et al*, 1994) and mammals (Dupont *et al*, 1994; Furukawa *et al*, 1998; Guennoun *et al*, 1995; Sanne and Krueger, 1995).

1.2.6. Aromatase

The conversion of androgens (i.e., androstenedione and testosterone) into estrogens is catalyzed by aromatase (Figure 2). The activity of this enzyme occurs in several tissues including the placenta (Fournet-Dulguerov *et al*, 1987), ovary (Lephart *et al*, 1995), adipocytes and testis (Simpson *et al*, 1989; Valladares and Payne, 1979). Moreover, molecular cloning of aromatase cDNA revealed the existence of a single enzyme in most species including trout (Tanaka *et al*, 1992), rat (Hickey *et al*, 1990), mouse (Terashima *et al*, 1991), chicken (McPhaul *et al*, 1988), bovine (Hinshelwood *et al*, 1993) and human (Harada, 1988). Furthermore, the presence of aromatase activity in CNS has been suggested from biochemical studies demonstrating androstenedione conversion into estrone, in the rat brain (Naftolin *et al*, 1972; Roselli *et al*, 1985). Immunocytochemical studies have shown that aromatase is expressed in neurons and in astrocytes but not in microglial cells (Garcia-Segura *et al*, 2003). Aromatase expression has been also evidenced in the human temporal cortex (Yague *et al*, 2006).

1.2.7. 5α-Reductase

The 5α-Rs are a family of isozymes expressed in a wide range of organs and tissues, including also the CNS. For definition, 5α-R is an enzyme that catalyses an irreversible reduction of a double bond at position 4.5 in specific steroids at 19 and 21 carbon atoms (C19 and C21). This occur through a NADPH-dependent mechanism or transferring a hydride ion from a nicotinamide adenine dinucleotide phosphate (NADPH) to a steroid precursor at 5α position, thus creating its corresponding 5α-reduced metabolite (Bramson *et al*, 1997).
In particular, this enzyme converts T into DHT, the PROG into DHP (Figure 2) as well as also the deoxycorticosterone (DOC) into 5α-dihydrodesossicorticosterone (DHDOC).
In the brain, these 5α-reduced metabolites are further metabolized by another enzymatic class, the 3α-HSOR or 3β-HSOR, in 3α-diol and 3β-diol in the case of T; THP and isopregnanolone in case of PROG, and 3α,5α-tetrahydrodesossicorticosterone (THDOC) in the case of DOC.

This reaction uses NADPH as a cofactor but in opposition to the previous one, it is a reversible process. Three isoforms of 5α-R, defined as type 1 (5α-R1), 2 (5α-R2) and 3 (5α-R3), have been cloned in humans and rats (Andersson and Russell, 1990; Berman and Russell, 1993; Paba *et al*, 2011).

In man, 5α-R type I (5α-R1) is mainly present in the sebaceous glands of many skin regions, including the scalp, and in the brain, liver, muscles (Ellis *et al*, 2005; Thigpen *et al*, 1993), but low levels were also found in the prostate (Titus *et al*, 2005). The isoform type II (5α-R2) is present in the brain, liver, epididymis, hair follicles, prostate and seminal vesicles (Thigpen *et al*, 1993). More recently, a type III (5α-R3) isoform, highly expressed in the skin, brain, mammary gland and breast cancer has also been described (Yamana *et al*, 2010). It is also important to note that, both in humans and rodents, the isoenzymes are expressed in different brain areas by neurons, astrocytes and glial cells (Castelli *et al*, 2013; Pelletier *et al*, 1994).

In rats, 5α-R1 and 5α-R2 cDNAs have been cloned from a prostate library but the two genes are transcribed in distinct cells: mRNAs encoding 5α-R1 are found in the basal epithelial cells, while 5α-R2 mRNAs are localized in stroma cells (Andersson *et al*, 1990; Berman *et al*, 1993). Unlike type 1 and 2, 5α-R type 3 is thought to play a role in protein glycosylation via the dolichol phosphate pathway (Garcia-Segura and Balthazart, 2009; Giatti *et al*, 2012b).

The 5α-R1 is responsible, approximately, for one third of the circulating DHT, while the remaining two thirds depend on the 5α-R2 (Gisleskog *et al*, 1998).

Comparing the structure of the rat protein with that of human, it emerges that the latter is composed of 259 amino acids and that the primary structure presents a 60% of homology with rat (Andersson *et al*, 1990). Furthermore, variants of the gene encoding this enzyme have been identified, especially missense mutations, which may alter for example the affinity of the enzyme for its substrates or for the NADPH cofactor. An example is the variant SRD5A2, which codes for the isoform II. The genes encoding 5α-R1, 5α-R2 and 5α-R3 are located in chromosome 5, 2 and 4, respectively, and the two isoenzymes have different optimal pH and sensitivity to substrates (Kahrizi *et al*, 2011; Normington and Russell, 1992; Uemura *et al*, 2008).

Moreover, no genetic deficiency has been reported for 5α-R1 enzyme even if the clinical consequences of congenital 5α-R2 deficiencies are well characterized and consist in alterations of sexual differentiation (Imperato-McGinley *et al*, 1974; Imperato-McGinley and Zhu, 2002; Katz *et al*, 1997; Russell and Wilson, 1994; Sasaki *et al*, 2003a; Sasaki *et al*, 2003b). Mutations in 5α-R3 have also been described and are associated with mental retardation and visual disturbances (Cantagrel *et al*, 2010; Kasapkara *et al*, 2012; Morava *et al*, 2010).

The 5α-R expression in the brain has been extensively studied (Melcangi *et al*, 1993; Pelletier *et al*, 1994; Saitoh *et al*, 1982; Stoffel-Wagner, 2003) and its activity has a crucial role in the physiological control of nervous function. The enzyme participates in the modulation of neuroactive steroids function in the brain, since it catalyses the formation of PROG and T metabolites that have the task

of modulating brain functions, such as behaviour and sexual differentiation, through the interaction with their receptors (Negri-Cesi *et al*, 2004).

In particular, the metabolites of PROG and T are involved in the regulation of neuroendocrine events, affection, learning and memory, synaptic and glial plasticity and adult hippocampal neurogenesis as well as in the response of brain tissues to injury and neurodegeneration (i.e., regulating neuronal survival, axonal regeneration and gliosis) (Brinton, 2013; Brunton *et al*, 2014; Kipp and Beyer, 2009; Panzica *et al*, 2012).

Moreover, 5α-R plays a fundamental role in neuroprotection, in the reparative actions of the central and peripheral nervous system, in the shelter of memory and learning functions in an experimental animal model of Alzheimer's, Parkinson's and multiple sclerosis (Caruso *et al*, 2008; Giatti *et al*, 2010; Labombarda *et al*, 2006; Melcangi *et al*, 2012; Pesaresi *et al*, 2006).

1.3. Mechanism of action of neuroactive steroids

1.3.1. Classical and non-classical receptors

As previously described, neuroactive steroids are actively metabolized by several enzymes, such as 5α-R, 3α-HSOR and 3β-HSOR. These enzymatic steps exert an important role in the mechanism of action of neuroactive steroids, since active metabolites of these molecules exert their effects by a variety of mechanism, including the activation of classical steroid receptors signaling mediated by progesterone receptor (PR), androgen receptor (AR) and estrogen receptors (ERs), which are present in different isoforms, such as A and B or α and β (see for review (Melcangi *et al*, 2008a). On the other hand, some of these metabolites may also exert their effects by binding to membrane receptors, such as GABA-A and GABA-B receptors, glutamate N-Methyl-D-aspartate (NMDA) receptor, AMPA and kainate subunits, membrane PROG receptors (mPRs) and pregnane X receptor (PXR) (Almey *et al*, 2014; Melcangi *et al*, 2008a; Nag and Mokha, 2014; Qin *et al*, 2015; Schumacher *et al*, 2014).

Notably, DHP as PROG, is able to bind the classical steroid receptor, the PR (Melcangi *et al*, 2008a), instead THP is a potent ligand of a non-classical steroid receptor, such as the GABA-A receptor (Belelli and Lambert, 2005; Lambert *et al*, 2003). However, only THP is able to bind and activate the GABA-A receptor, while isopregnanolone antagonizes the THP effect (Melcangi *et al*, 2008a). Similarly, DHT like its precursor T, interacts with AR, instead, 3α-diol is a GABA-A receptor agonist, while 3β-diol is an ERβ agonist (Handa *et al*, 2008; Melcangi *et al*, 2008a).

On the other hand, it is important to underline that also neuroactive steroid substrates, such as DHEA and PROG have been recently reported to exert effects via non-classical steroid mechanisms. For instance, in case of DHEA, it is unclear whether it is able or not to interact with AR (Lu *et al*, 2003; Mo *et al*, 2009; Mo *et al*, 2006) but available observations indicate a role for GABA-A receptors in DHEA signaling (Maninger *et al*, 2009). Moreover, in case of PROG, also in the nervous system, like in other tissues, classical PR may move to the cytoplasm or the plasma membrane and interacts with components of intracellular signaling pathways, such as kinases. This is also the case of ERs (Schumacher *et al*, 2014). Furthermore, picomolar concentrations of PREG sulfate are able to increase the intracellular response to glutamate at synaptic NMDA receptors via the phosphorylation of cAMP response element-binding protein (Smith *et al*, 2014). Thus, metabolic conversion of PROG and T into their metabolites may differently modulate the mechanism of action of their precursor molecules by recruiting specific pathways of central nervous system.

Both neurons and glial cells express the estrogenic receptors, represented by the two nuclear receptor isoforms, ERα and ERβ (Brinton *et al*, 2008; Melcangi *et al*, 2008a; Melcangi *et al*, 2001). Not all types of glial cells, however, express the same repertoire of steroid receptors and their expression varies depending on the state of activation of cells, localization and stage of development. For example, the ERα receptor is expressed in microglial cells whereas ERβ, AR and PR do not appear to be expressed in physiological conditions (Sierra *et al*, 2008; Sierra *et al*, 2007). Similarly, changes in expression and metabolism of steroid receptors in response to different insults have been observed. For example, in rats, brain lesions also induce the expression of ERα in vimentin and GFAP immunoreactive astrocytic cells, or the AR in microglia (García-Ovejero *et al*, 2002).

1.4. Effects of neuroactive steroids

Neuroactive steroids are involved in several physiological neuromodulatory actions. Their lack or decrease may lead to the onset of pathological changes. In agreement, their neuroprotective actions have been reported.

1.4.1. Physiological effects

One of the main roles performed is the involvement in sexual CNS differentiation during fetal life, such as morphological differentiation, size and number of neurons and glial cells in the brain as well

as in the spinal cord (Cooke *et al*, 1998). Furthermore, these molecules are not only involved in several molecular processes during fetal life but also in the post-natal period. In particular, they control sexual behavior, neuroendocrine regulation as well as the modulation of neurotransmitter release and expression of receptors for neurotransmitters themselves (Garcia-Segura *et al*, 1994). In particular, T that comes from gonads, acts in specific moments of fetal and post-natal life, inducing the onset of sexual dimorphism in the nervous system (Melcangi *et al*, 2008a). This hormone locally metabolized by neural tissue to estradiol from ARO or DHT from 5α-R, is especially important since the action of T-derivatives on both nuclear receptors leads to changes on the structural organization of nervous system (Melcangi *et al*., 2008a). In fact, the binding of both estradiol and DHT, to ER as well AR receptor, respectively, coordinates the appearance of typical male features in some brain areas as well as in spinal cord (Segovia *et al.,* 1999; Simerly, 2002). This binding ligand-receptor triggers several molecular mechanisms, involved in differentiation of size, morphology, number of neurons as well as glial cells, in neuronal and glial processes density, in neuropil and in the number of synapses both in males and in females (Cooke *et al*, 1998; Morris *et al*, 2004). This concept supports the idea that gonadal steroids do not act only on neurons, but also on glial cells, which actively participate in the metabolism of these steroids. In particular, glial cells are involved in the molecular processes that allow a dimorphic differentiation of neuronal connectivity (Garcia-Segura and Melcangi, 2006).

Furthermore, the glucocorticoids, the steroids linked to stress, influence underlie processes of development in CNS. In fact, stress and hormones related to it have effects on brain organization that may remain until adulthood. In particular, stress in the prenatal as well as perinatal period may alters the responses of the hypothalamic-pituitary-adrenal axis in adults leading to changes in the brain development, which may represent a risk factor for the onset of psychiatric and cognitive pathologies (Talge *et al*, 2007). In the adult brain, on the other hand, stress regulates cognitive abilities and anxiety rebound (Heim, & Nemeroff, 2001). These effects are associated with morphological and functional changes in different brain areas, including alterations in adult hippocampal neurogenesis (Mirescu *et al*, 2004) in the dendritic morphology of the amygdala (Vyas *et al*, 2004), in the prefrontal cortex (Cook and Wellman, 2004; Radley and Morrison, 2005) and in the hippocampus (Donohue *et al*, 2006; Sousa *et al*, 2000).

The effects produced by neuroactive steroids, occur by autocrine or paracrine actions that involve the control of gene expression through interaction with steroid nuclear receptors or through modulation neurotransmission, acting on membrane ion channels and neurotransmitter receptors. For instance, the PROG, produced by Schwann cells in response to the stimuli of adjacent neurons, regulates the

synthesis of myelin proteins through its receptor, the progesterone receptor (PR) (Plassart-Schiess and Baulieu, 2001).

The 5α, 3α-reduced metabolites of PROG (THP and 3α, 5β-tetrahydroprogesterone) are instead positive allosteric modulators of the GABA-A receptor. The activation by the latter, through hyperpolarization, increases the duration and the frequency of the chloride channel opening, which allows a low excitability of the neuronal membrane (Majewska, 1992). From a behavioral point of view, the modulation exerted through the control of the GABA-A receptor in the CNS leads to sedation, a lower anxiety and a reduced probability of neural action potential firing (Mellon *et al*, 2001).

THP regulates neuronal growth, survival and differentiation, causes the regression of neuronal terminations before they have established contacts with other neurons or glial cells, and protects neurons from the death induced by picrotoxin (Griffin *et al*, 2004). In contrast to 5α, 3α-reduced steroids derived from PROG, pregnenolone sulfate (PREGS) and DHEA sulfate (DHEAS) have antagonistic activity against the GABA-A receptor (Majewska, 1992). Its inhibition then causes states of anxiety and excitability (Trojnar *et al*, 2002). PREGS and DHEA, but not DHEAS, enhance the effect of NMDA on increasing intracellular calcium levels (Mellon *et al*., 2001). This mechanism may be responsible for the axonal growth induced by DHEA. DHEAS, on the other hand, promotes dendritic growth, even if the mechanism underlying this process has not yet been clarified (Mellon *et al*, 2001). Moreover, the steroid sulfate esters differentially affected the NMDA response since DHEAS is able to interact as a sigma agonist, that PREGS acts as sigma inverse agonist, and that PROG seems to perform a sigma antagonist effect (Monnet *et al*, 1995). The selective ligands of this receptor exert a neuromodulatory action on the excitatory neurotransmitter system, including the glutamatergic and cholinergic system. The modulation of NMDA-mediated glutamatergic neurotransmission through sigma1 receptor selective ligands plays a key role in major neuroadaptation phenomena, such as long-term potentiation, learning and memory, acute neuronal death and neurodegeneration (Maurice *et al*, 1999; Monnet *et al*, 1995). The neuromodulatory capacity of neuroactive steroids also includes their action on nicotinic, muscarinic, serotonergic, kainate and glycine receptors (Compagnone *et al*, 2000; Maurice *et al*, 1999; Stoffel-Wagner, 2001, 2003)

Neuroactive steroids are also able to regulate different functions of the peripheral nervous system, acting on both neurons and glial cells, in particular Schwann cells. Studies conducted in vivo, on the sciatic nerve of male rats, and in vitro, on cultures of rat Schwann cells, have shown that the synthesis of the protein zero (P0) and of the peripheral myelin protein of 22 kDa (PMP22), that is, the two most important myelin proteins produced by Schwann cells, is modulated by neuroactive

steroids. In particular, the expression of P0 increases following treatment with PROG and DHP, while that of PMP22 is induced only by THP (Melcangi *et al*, 2005; Melcangi *et al*, 1998; Melcangi *et al*, 1999).

Furthermore, the T and its metabolites influence the expression of these proteins, since it has been observed that the castration of adult male rats decreases the expression of P0 and PMP22 in the sciatic nerve. This reduction is reversed following treatment with DHT or 3α-diol in the case of P0 and with 3α-diol by PMP22 (Magnaghi *et al*, 2004; Magnaghi *et al*, 1999). A similar trend was also observed in cultures of rat-derived Schwann cells and treated with DHT or 3α-diol: DHT induces an increase in P0 expression (Magnaghi *et al*, 1999), while treatment with 3α-diol increases the mRNA levels of PMP22 (Melcangi *et al*, 2000).

These observations have suggested that the expression of the P0 protein may be under the control of the progesterone and androgen receptor, while a non-classical receptor, such as the one for GABA-A, may control the expression of PMP22, as well as THP and 3α-diol are modulators of this receptor (Melcangi *et al*, 2005). This hypothesis seems to be confirmed by studies using a series of selective inhibitors or agonists of PR, AR and GABA-A receptor (Magnaghi *et al*, 2007; Magnaghi *et al*, 2001; Melcangi *et al*, 2003; Melcangi *et al*, 2017) and by evidence that sequences putative responsive to PROG have been identified on the gene coding P0 (Magnaghi *et al*, 1999).

In addition to the important effects on myelin proteins, neuroactive steroids are also able to stimulate the expression of transcription factors involved in the myelination process (Guennoun *et al*, 2001; Magnaghi *et al*, 2007; Mercier *et al*, 2001), suggesting the ability of these molecules to control the process of myelination itself mediated by Schwann cells through different intracellular mechanisms. The axon compartment of the PNS are also influenced by neuroactive steroids. In particular, evidence obtained in vitro with co-cultures of neurons and Schwann cells has shown that PROG treatment is able to increase the expression of two important genes involved in the process of myelination, such as the small protein binding the GTP Ras-like (RAB-1) and the protein associated with phosphoribosyl diphosphate synthase (Chan *et al*, 2000). Moreover, during development, the use of mifepristone, a PR antagonist, causes a reduction in the axonal diameter compared to the thickness of myelin and, an increase in the density of neurofilaments (Melcangi *et al*, 2003).

1.4.2. Neuroprotective effects

Protective effects of neuroactive steroids have been reported in several neurodegenerative and psychiatric disorders, in which the levels of these molecules are altered.

For example, it has been shown that some brain areas of Alzheimer's disease (AD) patients, in addition to the accumulation of β-amyloid, have reduced levels of neuroactive steroids compared to the same regions from elderly people who do not present with the disease. In particular, decreased levels of DHEAS in hypothalamus and PREGS both in cerebellum and striatum were observed, which correlate with the increased concentrations of proteins involved in the formation of plaques and neurofibrillary aggregates, such as the β-amyloid and the phosphorylated Tau protein, typical of this pathology (Weill-Engerer *et al*, 2002). Moreover, subjects with AD demonstrate significant reductions in prefrontal cortex THP levels, a finding that may be relevant to neuropathological disease stage severity (Marx *et al*, 2006). Thus, restoring neuroactive steroid concentrations may represent a useful therapeutic approach for these neurodegenerative disorders. For example, THP promotes neurogenesis, improves learning and memory and ameliorated the pathology burden in the triple transgenic Alzheimer's disease (3 x TgAD) mouse model (Brinton, 2013; Irwin and Brinton, 2014).

The effects of neuroactive steroids were also evaluated on an experimental Parkinson's model, obtained using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which mimics in some aspects the human pathology (Vajda, 2002). The results obtained showed that the DHEA administration to MPTP-treated mice protects dopamine neurons in the striatum from neurotoxin (D'Astous *et al*, 2003). This is important because their reduced activity leads to neuronal death in the *pars compacta* of the substance nigra, which is considered the main cause of the appearance of Parkinson's disease in humans.

A recent~~ly~~ study (Morissette and Di Paolo, 2009) has shown that 1 month-treatment with 17β-estradiol is able to increase the levels of 3-methoxytiramine (dopamine metabolite) in the striatum of ovariectomized monkey, damaged by MPTP. These results are very important given that may mimic post-menopausal hormonal conditions.

Interesting results have also been obtained in the case of chronic manganese exposure (manganism, a neurodegenerative disease similar to Parkinson's disease), which leads to an altered glutamate transporter in astrocytes. As observed in primary cultures of cortical astrocytes, treatment with estradiol, as well as with a selective estrogen receptor modulator such as tamoxifen, increases

glutamate transport, also by increasing the expression of the growth factor (TGF-β1), and reverts the inhibition effects on manganese-induced glutamate uptake (Lee *et al*, 2011).

A peculiar neuroprotective action seems also to be performed by DHEAS. It has been shown that this steroid, but not equivalent doses of DHEA, is able to protect hippocampal neurons from glutamate-induced neurotoxicity (Mao and Barger, 1998). Accordingly, this specific neuroprotection DHEAS-induced was consistent with the increase of kappa-B dependent transcription factor activity, a hypothesis confirmed since the suppression of kappa-B binding to DNA prevents neuroprotective activity exerted by DHEAS.

Moreover, an effective neuroprotective action against ischemic damage seems to be performed by DHEAS. In fact, in an in vitro ischemia model (oxygen–glucose deprivation-induced injury in cerebellar granule cell culture), it protects cells, eliminating the characteristic apoptotic manifestations caused by the deprivation of both oxygen and glucose (Kaasik *et al*, 2001). The DHEAS neuroprotective action was inhibited by both pentobarbital and picrotoxin, the GABA-A receptor agonist and antagonist, respectively (Kaasik *et al*, 2001).

The neuroprotective effects exerted by PREG and DHEA seem to be due to their conversion to T and subsequently to estradiol, whose protective properties are the subject of an extensive literature (Behl, 2002; Brann *et al*, 2007). In fact, it has been demonstrated, for example, that these molecules are able to protect the hippocampal hilus neurons from death induced by kainic acid, in a dose-dependent manner, but that these effects are suppressed by the use of fadrozole, an inhibitor of ARO, which then blocks their conversion to estrogens (Veiga *et al*, 2003).

PROG and its metabolites may also have a neuroprotective effect through several mechanisms. For example, they may directly promote neuronal survival, by activation of signal transduction cascades associated with cell survival, such as the MAP kinase pathway and phosphoinositide 3-kinase (Kaur *et al*, 2007; Nilsen and Brinton, 2002; Singh, 2005), or by regulating the expression of pro-apoptotic and anti-apoptotic proteins (Djebaili *et al*, 2005; Nilsen *et al*, 2002; Yao *et al*, 2005). PROG also reduces oxidative stress (Ozacmak and Sayan, 2009) and lipid peroxidation (Roof and Hall, 2000). Furthermore, the 3α-THP metabolite may promote neuroprotection through inhibition of mitochondrial pore permeability (Sayeed *et al*, 2009). PROG and metabolites also exert a trophic action, due to their ability to regulate neurite growth and synaptic plasticity (Foy *et al*, 2008; Reyna-Neyra *et al*, 2002) or to promote neurogenesis under neurodegenerative conditions (Wang *et al*, 2008).

For these reasons, they may contribute to neuroprotective mechanisms, including the synthesis of neurotrophins, such as BDNF, which is increased by PROG in organotypic cultures of the cerebral

cortex (Kaur *et al*, 2007; Singh and Su, 2013; Su *et al*, 2012) and in the spinal cord (Gonzalez Deniselle *et al*, 2007; Gonzalez *et al*, 2005).

Furthermore, in the experimental model of multiple sclerosis (experimental autoimmune encephalomyelitis, EAE), PROG is not only able to decrease clinical severity, demyelination and neuronal dysfunction (Garay *et al*, 2008) but also to prevent axonal damage (Garay *et al*, 2009). In particular, PROG reduce inflammatory markers, such as microglial activation, IL-1β, TGF-β1 and increase the reduced activity of the Na^+, K^+ -ATPase pump (Giatti *et al*, 2012b). In particular, these effects appear to be due to the metabolism of PROG in DHP, as demonstrated by the increased levels of this steroid following PROG administration. Furthermore, PROG leads to increase axonal density and prevent the overexpression of GAP43 in the spinal cord of EAE animals. The fact that PROG supplementation may be protective in this experimental model is also supported since that a decrease in PROG and derivative levels has been observed in the spinal cord of EAE animals (Giatti *et al*, 2010). Another metabolite product, DHT, has also proved effective in reducing the severity of chronic disease in an EAE model: studies conducted by Giatti and colleagues (Giatti *et al*, 2015b) have shown that, 45 days after induction of the disease, DHT has a beneficial effect on neurological scores, accompanied by a decrease in gliosis and inflammation in the spinal cord. In addition, steroid administration improves some parameters associated with oxidative stress, tissue damage and mitochondrial activity. In addition, estradiol seems to be also protective in this model: its effect is expressed through the GPR30 receptor (coupled to proteins G), whose activation decreases the production of IL-17 following the increase of expression of the programmed death protein (PD-1) in $CD4^+$ $Foxp3^+$ regulatory cells, counteracting the clinical effects of the disease (Wang *et al*, 2009).

Interestingly, data related to psychiatric disorders have also been obtained. For example, in a model of reelin-haploinsufficiency (i.e. an experimental model for autism and schizophrenia), treatment with estradiol was able to increase the reduced number of Purkinje cells and the low levels of relin mRNA observed in the cerebellum of male mice. A particular profile of neuroactive steroids has also been noted, which occurs on the fifth day after birth in the cerebellum of these animals, i.e. there is both an increased T and estradiol levels, as well as a decrease in DHT levels (Biamonte *et al*, 2009).

In a randomized, placebo-controlled pilot study, it was shown that patients with schizophrenia or schizoaffective disorder receiving PREG show an important reduction in negative symptoms and improved cognitive function (Marx *et al*, 2009).

Neuroactive steroids play also an important neuroprotective action on the peripheral nervous system when occur alterations, such as neuropathies due to aging, physical damage and diabetes. Aging is a physiological situation that produces numerous biochemical and morphological alterations in the

peripheral nerves. It is in fact, associated with a decrease in the synthesis of P0 and PMP22, atrophy of the large myelinated fibers, increase in thickness of the myelin sheath and the generation of numerous abnormalities, such as myelin ballooning, splitting, infolding, reduplication and remyelination (Azcoitia *et al*, 2003; Melcangi *et al*, 2003). The PROG and DHP are able to increase the low levels of P0 expression in the sciatic nerve of elderly rats, while the expression of PMP22 is increased following treatment with THP (Melcangi *et al*, 1998; Melcangi *et al*, 1999). In addition to this effect on the expression of the main myelin proteins, these steroids are also able to improve the abnormalities of myelin itself. The protective effect of neuroactive steroids was also assessed in cases of nerve damage. Treatment with PROG or DHP is able to increase the low P0 gene expression levels in the distal portion of the sciatic nerve following nerve transection (Melcangi *et al*, 2000). It was also observed that the PROG and its precursor PREG, if administered locally, are able to balance the decrease in the number of myelinated membranes induced by cryotherapy of the sciatic nerve in a mouse model (Koenig *et al*, 1995). In addition to progestogens, T, DHT and DHEA have also demonstrated their effectiveness in peripheral nerve damage models (Ayhan *et al*, 2003; Huppenbauer *et al*, 2005). Diabetic neuropathy also causes alterations in the peripheral nerves. In a diabetic rat model induced with streptozotocin, it has been observed that treatment with PROG or DHP is able to counteract the increase in the number of fibers with alterations of myelin induced by the pathology at the level of the sciatic nerve. In the same model, it has been shown that PROG, DHP, THP, DHT and 3α-diol improve the alterations of the nerve conduction velocity and of the nociceptive thermal threshold. In addition, DHP, THP, T and its derivatives are able to restore intraepidermal fiber density, reduced in diabetes, while PROG, DHP and THP improve the Na^+, K^+ -ATPase pump activity.

1. 5. 5α-Reductase inhibitors: finasteride and dutasteride

The two most widely 5α-reductase inhibitors are dutasteride and finasteride (Bramson *et al*, 1997) (Figure 3). Dutasteride is a selective inhibitor of both 5α-R1 and 5α-R2, whereas finasteride, in humans, is primarily considered a 5α-R2 inhibitor, with a selectivity of about 100 times greater than 5α-R1 (Kenny *et al*, 1997; Russell *et al*, 1994). On the contrary, finasteride performs a comparable inhibition in both the 5α-R isoforms in rodents (Azzolina *et al*, 1997). Although, human 5α-R and the corresponding isoform in rat have about 60% homology compared to the primary sequence, the sensitivity to finasteride differs approximately 100-fold (Andersson *et al*, 1990).

Finasteride is a synthetic steroid that acts as an alternative substrate for the 5α-R enzyme, with which it forms an irreversible complex. The lipophilic structure of this drug, which mimics the steroid basic

structure, is therefore able to cross the blood-brain barrier and inhibit 5α-R in the CNS (Lephart, 1995).

In humans, pharmacokinetic data for 5α-R inhibitors are limited and generally reflect the results following a bolus administration of the drug (Guarna *et al*, 1998). Finasteride pharmacokinetics has been most extensively characterized following oral doses of either 1 or 5 mg/day in men, corresponding to treatment formulations for male pattern baldness (Propecia) and benign prostatic hyperplasia (BPH) (Proscar), respectively. A single 5 mg dose was found to produce peak plasma concentrations of 35–40 ng/mL (approximately 94 nM) finasteride within 2–6 h (Gormley *et al*, 1990; Guarna *et al*, 1995; Ohtawa *et al*, 1991; Stuart *et al*, 2001). When the pharmacokinetics of finasteride was examined after single and multiple administration of the drug over an increasing dose range (5–100 mg), the relationship between peak plasma concentration and dose of finasteride was linear (Ohtawa *et al.*, 1991). The relationship between finasteride dose and peak concentration did not change following multiple administration over 7 days, suggesting that no accumulation of finasteride occurred. The terminal half-life of finasteride in circulation, independent of dose, ranged from 4.7–7.1 h (Gormley, 1995).

Inhibition of Type II 5α-R blocks the peripheral conversion of testosterone to DHT, resulting in significant decreases in serum and tissue DHT concentrations. Treatment with finasteride produces a rapid reduction in serum DHT concentration from 60 to 80% (Carlin *et al*, 1992; Rittmaster, 1997), which demonstrates the contribution of the human type I enzyme to serum DHT levels. However, finasteride decreases prostatic DHT concentrations (where the type II enzyme predominates) by as much as 90% (Geller, 1990; Majewska, 1992).

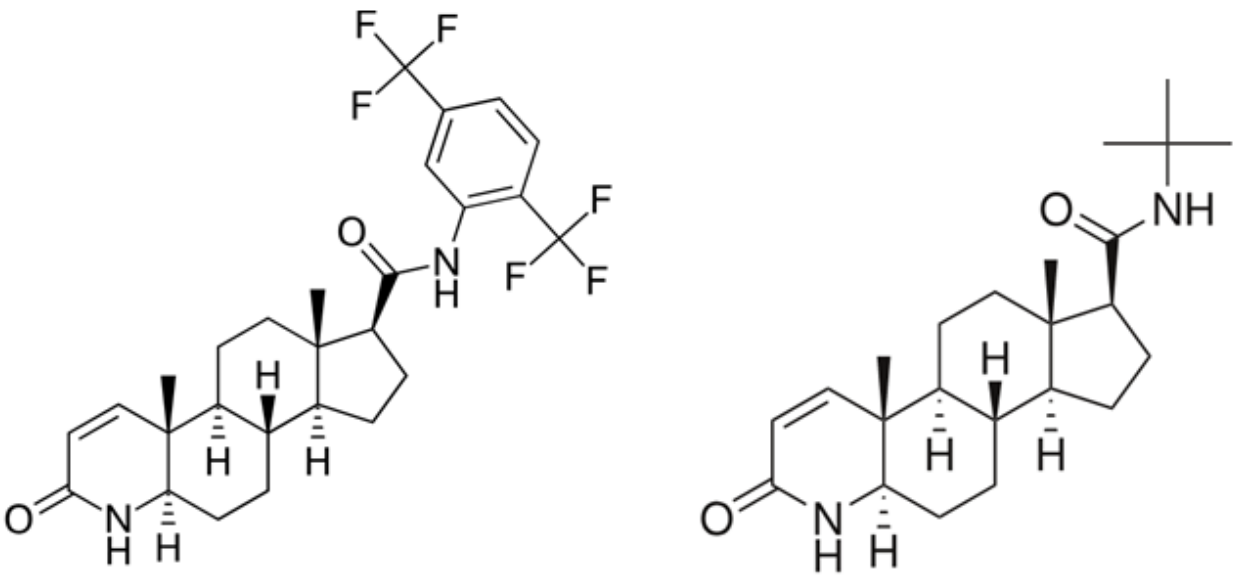

Figure 3. *Chemical structures of two 5α-reductase inhibitors (Dutasteride and Finasteride).*

Multiple daily doses for 1–2 weeks led to a similar 65–80% suppression of serum DHT (Gormley *et al*, 1990), suggesting that tolerance did not develop to a chronic finasteride regimen in men. It has been reported that DHT concentrations recover within 2-weeks following the cessation of finasteride treatment in men (Stoner, 1990), a finding that would be consistent with the slow turnover for the human type I and type II enzyme complexes (Moss *et al*, 1996).
Treatment with finasteride was approved by the Food and Drug Administration (FDA) for the treatment of benign prostatic hyperplasia (BPH) and hair loss (androgenic alopecia - AGA) in men. The drug is available on the USA market since 1992 for the treatment of BPH and since 1997 for AGA, with different posology.

AGA is a condition that occurs in men with an inherited sensitivity to the effects of androgens on scalp hair. It is characterized by visible loss of hair in areas of the scalp caused by progressive miniaturization of hair follicles. In addition, this disorder can affect both men and women, although the pattern differs between the two genus and the incidence is higher in men (Bienova *et al*, 2005). For the treatment of this disease, the optimal dose indicated is 1 mg/day; patients usually respond to therapy within 3 or 4 months, but treatment must continue for at least 12 months before assessing their efficacy (Roberts *et al*, 1999). Finasteride (Propecia) is a specific and competitive inhibitor of 5α-R II and has therefore a selective action on hair follicles. Scalp skin DHT levels fall by more than 60% after administration of finasteride, thereby suggesting that a significant amount of DHT found in scalp skin is derived from both local production and circulating DHT (Dallob *et al*, 1994). Thus, the effect of finasteride on scalp DHT is likely because of its effect on both local follicular and serum DHT levels. This explains why relatively small dose of finasteride may be adequate therapeutically.
In menopausal women with androgenic alopecia, treatment with finasteride is generally not effective (Olsen *et al*, 2005). In addition, finasteride is contraindicated for women in case of pregnancy, because it may lead to alterations to the reproductive tissues of a male fetus (Roy and Chatterjee, 1995). However, in women, this molecule has been used in some clinical studies for the treatment of hirsutism, in daily or intermittent doses of 2.5-5 mg (Cilotti *et al*, 2001).

The approved dose for the treatment of BPH is instead 5 mg (Logan and Belgeri, 2005) and several studies have observed a reduction in prostate volume of 19-27% (Andriole *et al*, 2004; Gormley, 1995; Rittmaster, 1997). Preliminary clinical studies have suggested that finasteride could be effective in the prevention of prostate cancer in humans. The use of finasteride as a chemopreventive agent, in a dose of 5 mg per day (as commonly prescribed for BPH), led to a 25% reduction in the probability of developing prostate cancer at the end of the 7-year clinical trial, when compared with subjects treated with placebo (Thompson *et al*, 2003).

As described by Hulin-Curtis et al., 2010 finasteride is both hydroxylated and oxidized by the enzymes belonging to the cytochrome P450 family (Hulin-Curtis *et al*, 2010). The second phase of metabolism consists instead of glucuronidation to allow an easier transport in the body. In the last phase of metabolism, it is excreted mostly through the gastrointestinal tract and minimally through the kidneys.

In humans, the pharmacokinetics of finasteride have been described in relation to doses of 1 or 5 mg/day, which correspond to the treatment of androgenic alopecia and benign prostatic hyperplasia (BPH). A single 5 mg dose is able to produce a peak in plasma concentrations of 35-40 ng/ml (about 94 nM) between 2 and 6 hours (Gormley *et al*, 1990; Ohtawa *et al*, 1991; Steiner, 1996). The half-life of the drug, regardless of the dose, varies from 4.7 to 7.1 hours (Gormley, 1995).

Treatment with finasteride produces a rapid reduction of the serum DHT concentration from 60 to 80% (Rittmaster, 1997), which demonstrates the contribution of the human type II enzyme to serum DHT levels. However, finasteride decreases the concentrations of prostatic DHT (where the type II enzyme is predominant) by as much as 90% (Geller, 1990; McConnell *et al*, 1992). It has been reported that DHT concentrations are generally recovered within 2 weeks after cessation of treatment with the drug (Stoner, 1990), and this would be consistent with the slow turnover of the enzyme complexes (Moss *et al*, 1996).

It has been reported in the literature that a chronic therapy with this drug may cause a series of side effects related to the sexual sphere, such as a libido reduction, erectile dysfunction, difficulty in achieving orgasm and ejaculatory disorders (Byrnes *et al*, 1995; Kaufman *et al*, 1998; Wessells *et al*, 2003). The studies carried out in this regard generally state that the incidence of these effects is very low, but they sometimes persist even after discontinuation of treatment (Irwig, 2012a, b; Irwig and Kolukula, 2011). Moreover, in addition to the symptoms just mentioned, some patients treated for androgenic alopecia may develop anxiety, depression, chronic fatigue and muscle pain during therapy, which may persist even after its suspension (Altomare and Capella, 2002; Melcangi *et al*, 2013a; Rahimi-Ardabili *et al*, 2006). This pathological condition is called Post-Finasteride Syndrome (PFS), and is associated with alterations in the levels of some neuroactive steroids found in the CSF and in plasma of patients (Caruso *et al*, 2015a; Melcangi *et al*, 2013a).

1.6. Post-Finasteride Syndrome (PFS)

During randomized controlled trials, necessary for registration, some rare side effects were observed in patients treated for BPH (Lowe *et al*, 2003). In particular, patients reported adverse sexual

symptoms, reversible upon drug withdrawal. In the post-marketing phase, however, it became clear that a few men continue to experience adverse events for long time (even years) later cessation of finasteride. This latter condition was also evident in young men treated for AGA (Irwig *et al*, 2011) who were assuming lower doses and with no co-morbidity related to the older age than BPH patients. These important side effects include sexual and psychiatric symptoms (e.g., depressed mood, suicidal thoughts), muscle pain and fasciculation (Irwig, 2012a; Traish *et al*, 2011). Moreover, patients reported bodily modifications reminding hypogonadism (e.g. decreased size of genital organs, breast tenderness, gynecomastia), as compared to the time before taking finasteride. Furthermore, a study performed by our research group discussed the knowledge accumulated so far on the pathological phenotype of this pathological condition, and in particular highlighting the possible common features on the sexual dysfunction differentiating the percentage of incidence of several parameters before, during and after treatment (Giatti *et al*, 2018a) (see Figure 4 for details).

However, it is important to highlight that the epidemiological dimension of the problem is currently unknown, indeed to date, the sum of these persistent symptoms, designated as the PFS, are not yet classifiable. Nevertheless, reporting of persistent sexual and depressive side effects in the finasteride post-marketing phase convinced the Swedish and the UK Medical Product Agencies, and also the Drug Italian Agency, AIFA (Agenzia Italiana del Farmaco) in 2017, to introduce a warning note in the patient information leaflet. The persistence of psychiatric symptoms after drug withdrawal is an intriguing aspect that, to the best of our knowledge, has been reported only in the case of treatment with phenothiazine, an antipsychotic drug, so far (Hunter *et al*, 1964).
These findings are a cause of concern, given severe morbidity and potentially related mortality, due to the high increase of depressive symptoms and suicidal thoughts. Although the rates of side effects may appear low or insignificant, their persistence and impact on the overall quality of life is not easily measured. The entity and seriousness of the symptoms, however, may be really distressing for the concerned patients, who are organized in self-help organizations (e.g. www.propeciahelp.com) seeking to rise the medical awareness to this condition and stimulate research on this peculiar matter.

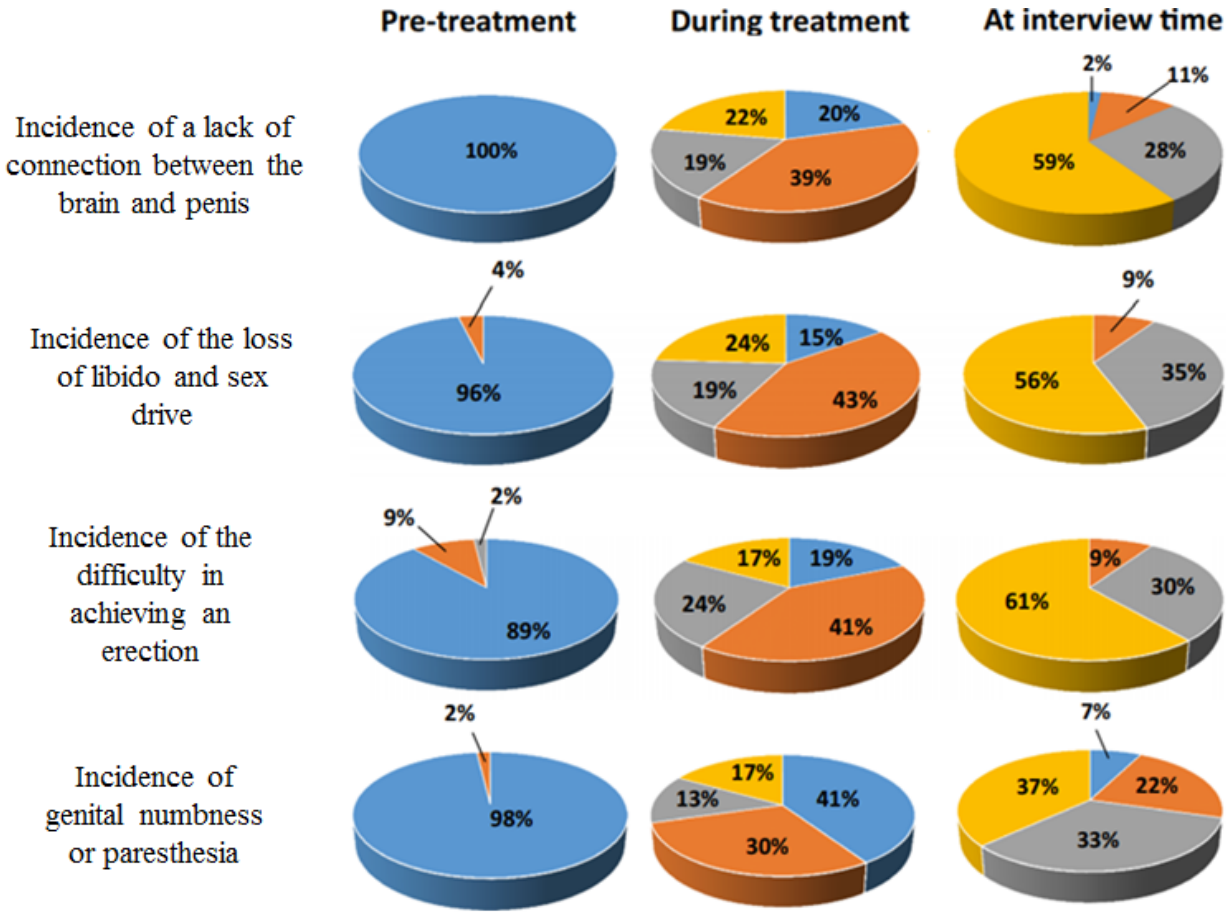

Figure 4. *Incidences of four parameters self-reported by PFS patients. Pie charts represent patients, expressed as percentage, reporting the frequency (blue: never; orange: sometimes; grey: often; yellow: always) of these symptoms before the treatment (pre-treatment), during the treatment and at interview time (i.e., at least three months after drug discontinuation; for further details, see review Giatti et al, 2018a. Figure modified by Giatti et al, 2018a.*

A relationship between T levels and depression has also been ascertained. Indeed, young hypogonadal as well as aged men, showing decreased levels of T, exhibit a high prevalence of anxiety disorders and major depression. Interestingly, it was observed that neuroactive steroid levels in plasma and CSF of three PFS patients were drastically altered, even years after finasteride suspension, in comparison to control subjects (Melcangi *et al*, 2013b) (See tables 1-2 for details).

In this study, it was shown for the first time, in three male patients with pattern hair loss, that anxious/depressive symptomatology and persistent sexual side effects despite suspension of finasteride are associated with changes in plasma and CSF levels of neuroactive steroids. In particular, in plasma, a decrease in DHP levels associated with an increase of 3a-diol and 17b-E was observed (Table 1).
On the contrary, in CSF we observed a decrease in metabolites of PROG and T, such as DHT, and isopregnanolone, associated with an increase in 17β-E and T (Table 2).

These preliminary observations highlight important changes in the levels of PROG metabolites: that could be very interesting since a role of 3a-reduced metabolites of PROG and in particular of THP is well established in anxious/depressive symptomatology. Indeed, there is general agreement that THP is decreased in CSF and plasma in patients with anxious/depressive symptomatology and that this disequilibrium may be reverted with different antidepressants (Caruso *et al*, 2013; Caruso *et al*, 2010; Uzunova *et al*, 2006; Zorumski *et al*, 2013).

Moreover, as self-reported by the patients (Altomare *et al*, 2002; Giatti *et al*, 2018a; Irwig, 2012a; 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).

		PREG	PROG	DHP	THP	Isopregnanolone	DHEA	T	DHT	3α-diol	3β-diol	17α-E	17β-E
PATIENTS (n=3)													
1		1.56	0.40	u.d.l.	u.d.l.	0.28	3.17	3.17	0.34	1.49	0.19	u.d.l.	0.05
2		2.48	0.64	u.d.l.	u.d.l.	0.58	20.1	7.94	0,44	2.51	0.12	u.d.l.	0.07
3		0.72	0.16	u.d.l.	u.d.l.	3.39	1.69	10.5	u.d.l.	u.d.l.	u.d.l.	u.d.l.	u.d.l.
	Mean	**1.59**	**0.40**	**u.d.l.**	**u.d.l.**	**1.42**	**8.32**	**7.2**	**0.28**	**1.35**	**0.12**	**u.d.l.**	**0.047**
	SEM	**0.51**	**0.14**			**0.99**	**5.9**	**2.15**	**0.12**	**0.71**	**0.04**		**0.001**
	Student's *t*-test			***						*			*
CONTROLS (n=5)													
1		1.10	0.47	0.27	0.10	0.11	3.15	6.69	0.53	0.05	0.05	u.d.l.	u.d.l.
2		0.87	0.35	0.32	0.10	0.10	1.28	5.80	0.53	0.05	0.05	u.d.l.	u.d.l.
3		1.29	0.40	0.31	0.10	0.11	4.21	12.04	0.63	0.05	0.05	u.d.l.	u.d.l.
4		0.83	0.24	0.25	0.22	1.28	7.16	13.90	0.31	0.05	0.18	u.d.l.	u.d.l.
5		1.36	0.15	0.22	0.14	0.51	4.36	5.86	0.22	0.07	0.53	u.d.l.	u.d.l.
	Mean	**1.09**	**0.32**	**0.27**	**0.13**	**0.42**	**4.03**	**8.86**	**0.44**	**0.05**	**0.17**	**u.d.l.**	**u.d.l.**
	SEM	**0.11**	**0.05**	**0.02**	**0.02**	**0.22**	**0.95**	**1.71**	**0.08**	**0.004**	**0.09**		

Table 1. *Levels of neuroactive steroids in plasma of three PFS patients and controls.*

*Data are expressed as pg/mL ±SEM. u.d.l. = under detection limit. *p < 0.05; ***p < 0.001 by Student's t-test.*

Mean and SEM values are in bold. Detection limit was 0.25 pg/mL for DHP; 0.1 pg/mL for THP; 0.05 pg/mL for DHT, 3a-diol and 3b-diol; 0.02 pg/mL for 17a-E and 17b-E.

PREG = pregnenolone, PROG = progesterone, DHP = dihydroprogesterone, THP = tetrahydroprogesterone, DHEA = dehydroepiandrosterone, T = testosterone, DHT = dihydrotestosterone, 3a-diol = 5a-androstane-3a,17b-diol, 3b-diol = 5a-androstane-3b,17b-diol, 17a-E = 17a-estradiol, 17b-E = 17b-estradiol, SEM =standard error of the mean. See Melcangi et al, 2013b for details.

		PREG	PROG	DHP	THP	Isopregnanolone	DHEA	T	DHT	3α-diol	3β-diol	17α-E	17β-E
PATIENTS (n=3)													
1		1.16	0.30	u.d.l.	u.d.l.	u.d.l.	0.24	0.07	0.29	0.26	u.d.l.	0.03	0.06
2		1.10	0.36	u.d.l.	u.d.l.	u.d.l.	0.38	0.25	0.35	0.29	u.d.l.	u.d.l.	0.06
3		0.23	0.11	u.d.l.	u.d.l.	u.d.l.	0.13	0.14	0.18	0.13	u.d.l.	u.d.l.	u.d.l.
	Mean	**0.83**	**0.26**	**u.d.l.**	**u.d.l.**	**u.d.l.**	**0.25**	**0.15**	**0.27**	**0.23**	**u.d.l.**	**0.023**	**0.047**
	SEM	**0.3**	**0.07**				**0.07**	**0.052**	**0.05**	**0.05**		**0.003**	**0.013**
	Student's t-test				**	*		*	*				*
CONTROLS (n=5)													
1		0.74	0.15	u.d.l.	0.32	1.11	0.12	0.07	0.49	0.23	u.d.l.	u.d.l.	u.d.l.
2		0.95	0.16	u.d.l.	0.34	0.38	0.18	0.08	0.27	0.44	u.d.l.	u.d.l.	u.d.l.
3		0.91	0.10	u.d.l.	0.77	0.44	0.14	0.05	0.80	0.44	u.d.l.	u.d.l.	u.d.l.
4		0.71	0.19	u.d.l.	0.51	0.15	0.16	0.09	1.26	0.38	u.d.l.	u.d.l.	u.d.l.
5		0.70	0.18	u.d.l.	0.45	0.49	0.16	0.09	1.00	0.23	u.d.l.	u.d.l.	u.d.l.
	Mean	**0.80**	**0.16**	**u.d.l.**	**0.48**	**0.51**	**0.15**	**0.08**	**0.76**	**0.34**	**u.d.l.**	**u.d.l.**	**u.d.l.**
	SEM	**0.05**	**0.002**		**0.08**	**0.16**	**0.01**	**0.007**	**0.18**	**0.05**			

Table 2 *Levels of neuroactive steroids in CSF of three PFS patients and controls.*

*Data are expressed as pg/mL ± SEM. u.d.l. = under detection limit. *p ≤ 0.05; **p < 0.01 by Student's t-test.*

Mean and SEM values are in bold. Detection limit was 0.25 pg/mL for DHP, 0.1 pg/mL for THP and isopregnanolone, 0.05 pg/mL for 3b-diol, 0.02 pg/mL for 17a-E and 17b-E.

PREG = pregnenolone, PROG = progesterone, DHP = dihydroprogesterone, THP = tetrahydroprogesterone, DHEA = dehydroepiandrosterone, T = testosterone, DHT = dihydrotestosterone, 3a-diol = 5a-androstane-3a,17b-diol, 3b-diol = 5a-androstane-3b,17b-diol, 17a-E = 17a-estradiol, 17b-E = 17b-estradiol, SEM = standard error of the mean. See Melcangi et al, 2013b for details.

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As reported by Basaria and collaborators, PFS patients have impaired sexual function as well as high depression scores (Basaria *et al*, 2016). In particular, it was established whether the persistent side effects of PFS patients are caused by androgen deficiency as well as a decrease in peripheral androgen action, or by a persistent inhibition of 5αR gene (SRD5A). Interestingly, they demonstrated that the body composition, strength, and nucleotide sequences of AR, SRD5A1 and SRD5A2 genes, were similar among symptomatic finasteride-user, asymptomatic finasteride-user and nonusers. Furthermore, T, DHT, other markers of peripheral androgen action, and expression levels of AR-dependent genes in skin did not differ among these three groups. However, it was demonstrated that the group of symptomatic finasteride-users had a higher depression scores an impaired sexual function, a more negative affectivity balance, and more cognitive complaints than the asymptomatic finasteride-user and nonusers. Thus, the PFS patients revealed depressed mood and abnormal function in brain circuitry linked to major depression and sexual arousal (Basaria *et al*, 2016).

Furthermore, as recently reported by Melcangi and collaborators, in sixteen PFS male patients, it was 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).

Indeed, it was confirmed the presence of persistent ED and MDD in PFS patients, as previously shown by the recent results of Basaria and colleagues who found functional MRI abnormalities in brain regions of PFS patients targeted by the dopamine system (e.g. nucleus accumbens and prefrontal cortex) that are critical for normal erectile function and overlap with abnormalities seen in MDD (Basaria *et al*, 2016). Interestingly, self-reported occurrence of persistent ED after finasteride withdrawal in young patients treated for AGA who had no prior history of ED before finasteride treatment. However, contrary to previous results, it does not find a decrease in T plasma levels, suggesting that other neuroactive steroids could be responsible for ED (Table 3) (Melcangi *et al*, 2017).

In the plasma of PFS patients, it was reported a decrease of THP levels (Table 3). A THP level decreased is a common feature of anxious/depressive symptomatology and this disequilibrium may be corrected by antidepressant (Romeo *et al*, 1998; Zorumski *et al*, 2013).

However, in this group of PFS patients, an increase in T levels were detected both in plasma and in CSF (Table 3). In this context it is important to highlight that, the CSF levels of the active metabolite of T, DHT (i.e., a neuroactive steroid showing, in comparison to T, higher affinity vs androgen receptor) were significantly decreased in all PFS patients (Table 3). Thus, it was considered that T levels may not be predictive of ED and MDD, while its metabolite seems to be related to these conditions. Moreover, an involvement of AR in PFS effects has been already proposed. In fact, an

upregulation of AR expression in the prepuce of PFS patients (Di Loreto *et al*, 2014) and in the nervous system of male rats (Giatti *et al*, 2016a) one month after end of treatment with finasteride (i.e., withdrawal period) was observed. Thus, a combination between reduction in DHT and upregulation of AR could be proposed as pathogenetic mechanism underlying PFS (Melcangi *et al*, 2017).

Administration of finasteride seems to affect some of the cited aspects also in animals. Firstly, there is a growing number of basic scientific evidence that finasteride reduces the concentrations of several neuroactive steroids important for neurogenesis and neuronal survival. Several studies suggested a link between inhibition of 5α-R to symptoms of depression and this may be related to decreased production of reduced metabolites of T and PROG in the brain (Altomare *et al*, 2002; Duskova *et al*, 2009; Finn *et al*, 2006; Romer *et al*, 2010). Moreover, studies in animal models showed that acute injection of finasteride induces behavioral changes such as anxiety (open field) and depression (forced swim test) of cycling rats (Frye and Walf, 2002). Furthermore, male mice sub-chronically treated with finasteride had reversibly lower levels of brain DHT and less neurogenesis in the hippocampus (Romer *et al*, 2010). Likewise, finasteride lowers the concentration of ALLO that protects neurons from apoptosis via the Bcl-2 and Bcl-xL genes (Charalampopoulos *et al*, 2004). Finally, the decreased levels of neuroactive steroids in the hypothalamus and limbic areas (i.e., medial preoptic area, amygdala, and nucleus accumbens) may affect brain dopaminergic systems related to sexual desire (Pfaus *et al*, 2010).
As mentioned above, depressive symptoms are also correlated with increased inflammatory markers, and lack of effectiveness of antidepressants seems to be related to an overall activation of the inflammatory system (Carvalho *et al*, 2013). Administration of neuroactive steroids, like PROG, DHP and estrogens, is effective in reducing inflammatory parameter in many pathological situations (Giatti *et al*, 2012b). Even if there is no a direct correlation between finasteride administration and mitochondrial functions, it is known that mitochondrial failure could compromise neural plasticity, leading ultimately to depression. On the other hand, androgens seem to affect mitochondria (Holmes *et al*, 2013), improving their functions; so it is possible to speculate that the lack of DHT may be detrimental for the energetic machinery of the cell. Finally, reduced levels of trophic factors were observed in depressed patients, and it has been reported in experimental models that finasteride and androgen deprivation can alter brain derived neurotrophic factor functions (Hill *et al*, 2012; Morita and Her, 2008). Altogether these observations suggest a link between finasteride, depressive symptomatology and neuroactive steroid levels.

	PREG	PROG	DHP	Isopreg	THP	DHEA	T	DHT	3α-diol	3β-diol	17β-E
CSF											
CTRL (N=25)	0.31±0.21	0.19±0.14	2.83±1.86	0.11±0.03	2.01±4.23	0.2±0.11	0.13±0.11	0.25±0.21	UDL	UDL	0.07±0.05
PFS (N=14)	0.12±0.11 ***	UDL ***	0.56±0.90 ***	UDL	0.18±0.21	0.33±0.07 ***	1.97±1.99 ***	0.06±0.01 ***	0.20±0.43 *	UDL	UDL **
PLASMA											
CTRL (N=25)	0.96±0.84	0.18±0.09	0.73±1.44	0.42±1.56	0.29±0.49	1.86±3.19	5.70±2.75	0.42±0.39	0.12±0.12	0.17±0.25	UDL
PFS (N=14)	2.48±2.02 **	0.20±0.23	0.26±0.04 *	0.26±0.41	UDL **	8.79±7.42 **	12.3±3.99 ***	0.49±0.21	0.18±0.13	0.12±0.16	UDL

Table 3. *Neuroactive steroid levels in cerebrospinal fluid and plasma in controls and PFS patients. Data are expressed as pg/μL (mean ± SD). UDL = under detection limit. *p < 0.05, **p < 0.01 and ***p < 0.001 vs CTRL by Mann-Whitney U test. Detection limit was 0.1 pg/μL for Isopreg and THP, 0.05 pg/μL for PROG, 3α-diol and 3β-diol, 0.02 pg/μL for 17β-E.*

PREG = pregnenolone, PROG = progesterone, DHP = dihydroprogesterone, Isopreg = isopregnanolone, THP = tetrahydroprogesterone, DHEA = dehydroepiandrosterone, T = testosterone, DHT = dihydrotestosterone, 3α-diol = 5α-androstane-3α,17β-diol, 3β-diol = 5α-androstane-3β,17β-diol, 17β-E = 17β-estradiol. See Melcangi et al, 2017 for details.

1.7. Experimental observations with finasteride

The clinical applications concern the ability of finasteride to inhibit the type II isoform of the enzyme 5α-R, which is the predominant form in the prostate and in human hair follicles, and the concomitant inhibition of T-reduction in DHT. As mentioned above, both 5α-reductase isoforms are responsible for the reduction of PROG and DOC into DHP and DHDOC, respectively. Preclinical data indicate that the subsequent 3α-reduction of DHT, DHP and DHDOC produces steroid metabolites with rapid non-genomic effects on brain function and behavior, primarily through enhanced GABAergic inhibitory transmission. Consistent with their ability to enhance the action of GABA on GABA-A receptor, these steroid derivatives have anticonvulsant, antidepressive and anxiolytic actions, as well as modifying aspects of sexual and alcohol-related behavior (Finn *et al*, 2006). Thus, finasteride, which inhibits both 5α-reductase isoforms in rodents, has been used as a tool to manipulate levels of neuroactive steroids and determine their impact on behavior.

Finasteride was used to study anxiety and depression in experimental models. Numerous studies have shown that systemic administration of 3α, 5α-THP produces anxiolytic and antidepressant effects (Gasior *et al*, 1999; Khisti *et al*, 2000; Reddy *et al*, 2005). Co-administration of a 5α-R inhibitor with PROG blocks the anxiolytic effect, demonstrating that the effect depends on the conversion of PROG into its 5α, 3α-reduced metabolite (Bitran *et al*, 1995). A similar strategy has been used to demonstrate that the anxiolytic effect of T is mediated by its 5α-reduced metabolite, DHT (Edinger and Frye, 2004).

Bilateral THP infusion in the dorsal hippocampus (Bitran *et al*, 1999) or in the amygdala (Brot *et al*, 1997) produces anxiolytic effects, whereas intracerebroventricular administration causes antidepressant effects (Hirani *et al*, 2002). In contrast, the administration of both systemic and intra-hippocampal finasteride increases depression-like behaviors and anxiety in the rat (Frye *et al*, 2002; Frye and Walf, 2004). A subsequent study has also shown that the drug induces the same effects even when administered directly in the amygdala (Walf *et al*, 2006).

In an animal model of premenstrual dysphoric disorder (Smith *et al*, 2006) it is observed that three daily injections of finasteride significantly reduce THP levels in the hippocampus. This reduction is associated with the decrease in sensitivity to the anxiolytic effects of 3α, 5β-THP (the 5β isomer of THP), which is a positive modulator of the GABA-A receptor, slightly less potent than 3α, 5α-THP (Wang, 2011).

At the preclinical level, finasteride has proved to be a useful tool to clarify the role of neuroactive steroids in epileptic activity. Although the exact mechanism by which neuroactive steroids reduce the severity of discharges is not yet well understood, the anticonvulsant action of the metabolites depends on their ability to rapidly increase GABAergic neurotransmission. Although both PROG

and THP are positive modulators of the GABA-A receptor (Gee *et al*, 1995; Paul *et al*, 1992) THP is 500 times more potent than its precursor (Majewska, 1992), and some studies performed in models animals, using finasteride, have shown that it is precisely THP, more than PROG, that modulates seizure severity (Kokate *et al*, 1999).

There is an inverse relationship between endogenous THP and susceptibility to epileptic attacks in female rats. In one study (Frye *et al*, 1998a), the hormonal pro-estrogen phase was mimicked in ovariectomized rats (OVX) by the administration of high doses of PROG. In this animal model there is an increase in THP levels in the brain and a fall in the seizure is due to stimulation of the perforating pathway. The administration of finasteride reverses the effects just described, suggesting that the metabolism of PROG to THP contributes to the anticonvulsant effects of the estrous phase. Finasteride is also able to reduce the anticonvulsant effects of PROG and fluoxetine in male mice treated with pentilentetrazole (PTZ) (Kokate *et al*, 1999; Ugale *et al*, 2004) and of PROG in KO mice for PR.

Even in a type of epilepsy called catamenial (typical of the woman during the menstrual cycle), the administration of finasteride to pseudo-pregnant rats, with high levels of PROG, produces a significant increase in PTZ-induced convulsions, compared to controls (Reddy and Rogawski, 2000, 2001). These studies have been useful to demonstrate that the anticonvulsant effect of PROG is not mediated by the link with PR, and therefore by the PROG itself, but its metabolism is necessary.

Considering the metabolism of T, pro-or anticonvulsant properties were observed, depending on whether the T was aromatized or reduced. In fact, by blocking the aromatization of 17β-estradiol T with letrazol, the proconvulsive effect is eliminated; on the contrary, Finasteride is able to eliminate the anticonvulsant effect, which therefore depends on a 5α-reduced metabolite of T, 3α-androstenediol (Frye and Reed, 1998b).

Finasteride has also been used to study some aspects of the sexual behavior of female rats, such as lordosis. The lordosis is the typical measure of sexual activity in the female rats: they assume a position of sexual receptivity, with the arched back and bent tail, which allows the coupling with the male rodent. For this activity is necessary a release of estrogen in the CNS, followed then by a release of progestogens. These steroids modulate sexual receptivity in rodents primarily through actions on classical intracellular steroid receptors, but these genomic effects may also be coupled to non-genomic effects (Frye and Vongher, 2001). For example, THP can influence sexual behavior through its interaction with the GABA receptor.

The pharmacological manipulation of 5α-reduced metabolites also provided important insights to elucidate the role of neurosteroids in alcohol-related behaviors and in ethanol dependence. In fact, alcohol interacts with various neurotransmitter systems, including the GABAergic system (Grobin *et*

al, 1998). The effect of finasteride in this context seem to be mediated above all by THP. Different doses of THP are able to influence the behavior of mice and male rats in ethanol intake (Janak *et al*, 1998). Furthermore, infusions of ethyl alcohol in doses ranging from 1 to 4 g/kg significantly increase the levels of THP and THDOC in plasma, cortex and hippocampus (Barbaccia *et al*, 2001). Pre-treatment with finasteride reduces the levels of THP in the cerebral cortex in male rats (VanDoren *et al*, 2000) and leads male mice to a lower consumption of ethanol (Ford *et al*, 2005). In addition, VanDoren and colleagues demonstrate, through electrophysiological studies, that pretreatment with the drug prevents the alcohol-induced discharge of spontaneous discharges into the neurons of the medial septum of Broca's area (VanDoren *et al*, 2000).

A study carried out by Römer and colleagues (Romer *et al*, 2010) focused on the effects of finasteride on neuronal plasticity. In this work, treatment with the drug significantly reduces, in a mouse model, the brain levels of DHT and adult hippocampal neurogenesis, which is a crucial mechanism in adapting to environmental stimuli. In castrated animals, substitution therapy with T or DHT may increase cell survival in the hippocampus. Furthermore, they have shown that finasteride is able to cause structural changes in the limbic system, an area of the CNS that is fundamental for emotional behavior.

To all these reasons, many researchers have used an inhibitor of a rate-limiting step of steroid metabolites as a pharmacological tool to study their effects in the central and in peripheral nervous system. However, despite of the wide therapeutic use of finasteride as well as a consistent use in pre-clinic research, studies about the effects of finasteride treatment *per se* in CNS did not never conducted. Only recently, Giatti and collaborators, have conducted new experiments in an animal model to analyse the impact of finasteride treatment on neuroactive steroids in an experimental model (Giatti *et al*, 2016b).

Therefore, the effects of a subchronic treatment with finasteride at low doses (3 mg/kg/day) (Table 4) and the consequences of its withdrawal (Table 5) on neuroactive steroid levels in plasma, cerebrospinal fluid and some brain regions have been evaluated.

Finasteride treatment did not significantly affect the levels of PROG and T in plasma, the CSF and brain tissues, with the exception of the hippocampus, in which, as expected, the 5α-R inhibitor caused a significant increase in the levels of PROG, the substrate of the enzyme. However, the increase in the levels of PROG was not accompanied by a decrease in DHP levels (Table 4).

Concerning the 5α-R products, we detected a decrease in DHT levels in plasma after finasteride treatment, in agreement with what is observed in patients treated with this drug (Duskova *et al*, 2010).

	PLASMA		CSF		CEREBRAL CORTEX		CEREBELLUM		HIPPOCAMPUS	
	Control	Finasteride	Control	Finasteride	Control	Finasteride	Control	Finasteride	Control	Finasteride
PREG	0.20 ± 0.046	0.19 ± 0.03	0.31 ± 0.091	0.53 ± 0.07 *	1.70 ± 0.24	2.01 ± 0.23	1.60 ± 0.45	1.08 ± 0.29	5.61 ± 1.25	8.68 ± 1.63
PROG	1.31 ± 0.218	0.94 ± 0.12	0.62 ± 0.07	0.53 ± 0.035	2.61 ± 0.68	2.94 ± 0.31	0.58 ± 0.16	0.98 ± 0.24	6.26 ± 0.44	9.64 ± 1.45 *
DHP	0.56 ± 0.07	0.52 ± 0.14	0.49 ± 0.12	0.31 ± 0.06	5.18 ± 0.81	5.49 ± 0.76	2.94 ± 1.06	5.56 ±0.89 *	9.40 ± 2.53	10.3 ± 1.48
ISOPREG	u.d.l.	0.14 ± 0.01 *	0.13 ± 0.022	0.11 ± 0.007	2.54 ± 0.62	3.48 ± 1.03	0.51 ±0.06	1.16 ± 0.20 **	0.84 ± 0.11	0.84 ± 0.27
THP	0.25 ± 0.07	0.14 ± 0.04	2.31 ± 0.15	2.23 ± 0.15	15.6 ± 1.75	19.0 ± 1.95	5.34 ± 0.48	9.34 ± 2.33	u.d.l.	u.d.l.
DHEA	0.11 ± 0.03	0.11 ± 0.04	0.096 ± 0.02	0.060 ± 0.004	0.81 ± 0.11	0.48 ± 0.06 **	0.51 ± 0.08	0.75 ± 0.19	0.29 ± 0.09	0.30 ± 0.08
T	4.23 ± 1.19	3.16 ± 0.91	0.10 ± 0.01	0.12 ± 0.037	2.38 ± 0.37	1.71 ± 0.32	1.86 ± 0.26	1.89 ± 0.26	3.21 ± 0.56	1.97 ± 0.78
DHT	0.79 ± 0.09	0.30 ± 0.02 ***	1.54 ± 0.12	1.65 ± 0.20	17.5 ± 1.89	16.9 ± 1.55	0.83 ± 0.50	1.08 ± 0.11	3.87 ± 0.63	4.00 ± 0.41
3α-diol	0.84 ± 0.10	1.04 ± 0.28	0.076 ± 0.014	u.d.l.*	0.57 ± 0.06	0.60 ± 0.11	0.54 ± 0.07	0.34 ± 0.07 *	0.26 ± 0.07	0.30 ± 0.05
3β-diol	u.d.l.	u.d.l.	0.056 ± 0.004	0.10 ± 0.02 *	0.79 ± 0.17	0.60 ± 0.08	0.25 ± 0.034	0.72 ± 0.21 *	u.d.l.	u.d.l.
17α-E	u.d.l.	u.d.l.	u.d.l.	u.d.l.	0.076 ± 0.02	0.053 ± 0.01	0.054 ± 0.02	0.077 ± 0.02	u.d.l.	u.d.l.
17β-E	0.022 ± 0.001	0.048 ± 0.01 *	0.06 ± 0.02	0.04 ± 0.012	0.15 ± 0.03	0.10 ± 0.01	0.032 ± 0.006	0.066 ± 0.02 *	0.03 ± 0.007	0.05 ± 0.008

Table 4 *Levels of neuroactive steroids in plasma, CSF and brain areas of control and finasteride-treated male rats: effects after subchronic treatment.*

Data (n = 8 rats for each group) are expressed as pg/μl ± SEM in case of plasma and CSF and pg/mg ± SEM in case of brain areas. u.d.l. = Under detection limit.

*Student's t test analysis: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs control.*

Detection limits were 0.05 pg/μl or pg/mg for 3β-diol, 0.02 pg/l or pg/mg for 17α-E, 0.1 pg/μl or pg/mg for ISOPREG and THP. See Giatti et al, 2016b for details.

However, DHT levels in the CSF and brain tissue were not affected by the subchronic finasteride treatment (Table 4). Finasteride treatment did not significantly affect DHP levels in the other brain areas considered. Thus, seems that the subchronic treatment with a low dose of finasteride does not have major effects on the levels of the 5α-R substrates and products in the brain of male rats but that it is only able to affect peripheral DHT production.
However, finasteride caused important changes in the levels of other neuroactive steroids, such as ISOPREG, DHT and 17β-E in plasma, PREG, 3α-diol and 3β-diol in the CSF, DHEA in the cerebral cortex and ISOPREG, 3α-diol, 3β-diol and 17β-E in the cerebellum. These changes, involving secondary metabolites of PROG and T, as well as PROG and T precursors, show regional specificity and suggest that 5α-R inhibition impacts on other steroidogenic steps (Giatti *et al*, 2016b)

As concern the analysis of neuroactive steroids, one month after finasteride withdrawal, long-term consequences were revealed. In the plasma of finasteride-treated animals, it was detected a significant decrease in the levels of THP, PROG, DHT and 3α-diol compared to control animals (Table 5). Moreover, in the CSF, the levels of T and 17β-E were increased, while the levels of DHT were decreased, suggesting an increased accumulation of T and an increased conversion of T into 17β-E, caused by the inhibition of the conversion of T into DHT (Table 5). Interestingly, post-finasteride patients with persistent symptomatology showed significant decreased THP levels in plasma, as well as increased T levels in CSF that were associated with a decrease in DHT levels (Caruso *et al*, 2015b). Stronger effects at the end of the withdrawal period were detected in the cerebral cortex, in which the levels of PROG, DHP, ISOPREG, THP, DHEA and 17β-E were significantly decreased (Table 5). In the hippocampus, the PREG and PROG levels were decreased, while the levels of DHP were increased (Table 5). These outcomes indicate that finasteride treatment has long-term effects on neuroactive steroids the levels, even after a long period of withdrawal.
Indeed, the effects of finasteride withdrawal were stronger than the effects of finasteride treatment *per se*, in particular in brain regions such as the hippocampus and the cerebral cortex.
This may suggest that the steroidogenic system leads to partial compensation of the chronic inhibition of 5α-R activity. However, after this adaptation, the system may be less flexible to adapt and compensate for a new alteration and could be further disrupted when 5α-R activity is recovered (Giatti *et al*, 2016b).

	PLASMA		CSF		CEREBRAL CORTEX		CEREBELLUM		HIPPOCAMPUS	
	Control	Finasteride	Control	Finasteride	Control	Finasteride	Control	Finasteride	Control	FInasteride
PREG	0.19 ± 0.03	0.18 ± 0.04	0.20 ± 0.051	0.27 ± 0.04	1.07 ± 0.48	1.24 ± 0.18	0.66 ± 0.03	1.06 ± 0.12 **	7.44 ± 1.07	4.37 ± 1.35 *
PROG	2.12 ± 0.35	1.32 ± 0.21 *	0.64 ± 0.06	0.57 ± 0.05	3.08 ± 0.44	1.83 ± 0.31 *	1.48 ± 0.30	1.52 ± 0.57	8.44 ± 1.07	5.27 ± 0.56 *
DHP	0.32 ± 0.03	0.34 ± 0.04	0.45 ± 0.12	0.56 ± 0.07	12.03 ± 1.57	3.52 ± 0.21 ***	2.59 ± 0.61	4.38 ±0.70 *	6.67 ± 0.72	9.88 ± 1.27 *
ISOPREG	u.d.l.	0.13 ± 0.04	0.11 ± 0.004	0.11 ± 0.004	2.93 ± 0.35	1.89 ± 0.32 *	0.65 ±0.08	0.88 ± 0.16	0.47 ± 0.06	0.59 ± 0.10
THP	0.50 ± 0.09	0.14 ± 0.03 **	2.49 ± 0.19	2.29 ± 0.19	17.8 ± 1.75	7.5 ± 0.47 ***	4.12± 0.37	4.58 ± 0.63	u.d.l.	u.d.l.
DHEA	u.d.l.	u.d.l.	0.064 ± 0.007	0.14± 0.03 *	1.26 ± 0.31	0.30 ± 0.04 **	0.53 ± 0.10	0.40 ± 0.09	0.95 ± 0.43	0.29 ± 0.08
T	3.30 ± 0.7	3.31 ± 0.78	0.15 ± 0.03	0.30 ± 0.07 *	3.89 ± 0.61	3.65 ± 0.63	1.22 ± 0.27	2.26 ± 0.25 **	7.56 ± 2.70	4.84 ± 1.14
DHT	0.44 ± 0.04	0.33 ± 0.04 *	1.35 ± 0.04	0.98 ± 0.10 **	9.73 ± 1.38	9.00 ± 0.59	0.60 ± 0.16	0.23 ± 0.12 *	3.03 ± 0.43	4.00 ± 0.37
3α-diol	1.63 ± 0.29	1.00 ± 0.19 *	u.d.l.	u.d.l.	0.64 ± 0.18	0.52 ± 0.041	0.49 ± 0.14	0.31 ± 0.08	0.26 ± 0.03	0.23 ± 0.04
3β-diol	u.d.l.	u.d.l.	u.d.l.	u.d.l.	0.52 ± 0.21	0.53 ± 0.09	0.63 ± 0.18	0.19 ± 0.03 *	u.d.l.	u.d.l.
17α-E	u.d.l.	u.d.l.	u.d.l.	u.d.l.	0.046± 0.009	0.045 ± 0.009	0.064 ± 0.03	0.041 ± 0.03	u.d.l.	u.d.l.
17β-E	0.028 ± 0.004	0.024 ± 0.002	u.d.l.	0.04 ± 0.009 **	0.12 ± 0.02	0.06 ± 0.01 *	0.037 ± 0.01	0.055 ± 0.01	0.074 ± 0.02	0.034 ± 0.01

Table 5 *Levels of neuroactive steroids in plasma, CSF and brain areas of control and finasteride-treated male rats: effects at 1 month of withdrawal.*

Data (n = 8 rats for each group) are expressed as pg/μl ± SEM in case of plasma and CSF and pg/mg ± SEM in case of brain areas. u.d.l. = Under detection limit.

*Student's t test analysis: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs control.*

Detection limits were 0.05 pg/μl or pg/mg for DHEA, 3α-diol and 3β-diol, 0.02 pg/l or pg/mg for 17α-E and 17β-E, 0.1 pg/μl or pg/mg for ISOPREG and THP. See Giatti et al, 2016b for details.

In addition, in this study the expression of classical and non-classical steroid receptors has been assessed in male rats. For instance, in the cerebral cortex, in which it was detected decreased levels of DHEA both after finasteride treatment and at the withdrawal, it was detected an upregulation of AR expression. Even if the mechanism of action of DHEA in the nervous system has not yet been fully characterized, some observations suggest an interaction with AR (Lu *et al*, 2003; Mo *et al*, 2006). In addition, in the cerebral cortex, the decreased levels of 17β-E after finasteride withdrawal was associated with the upregulation of ERα (Giatti *et al*, 2016b). In contrast, the expression of ERβ was decreased. This finding suggests that the expression of ERα and that of ERβ are regulated by different mechanisms in the cerebral cortex as previously observed in aged male rats, in cerebral cortex (Munetomo *et al*, 2015).

As concern non-classical receptors the levels of ISOPREG, which in contrast to THP does not bind directly to the GABA-A receptor but, as mentioned before, can antagonize the effects of THP on the GABA-A receptor (Bengtsson *et al*, 2016; Hedstrom *et al*, 2009; Melcangi *et al*, 2008b) were also decreased in the cerebral cortex at withdrawal and this was also associated with a decrease in the gene expression of α4 and β3 subunits of GABA receptors (Giatti *et al*, 2016a).

1.8. Depressive symptomatology

Depressive disorders are complex, multifactorial mental disorders with unknown neurobiology, 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 both neurogenesis and neuron survival with mitochondrial impairment (Blier, 2013; Masi and Brovedani, 2011).

However, the molecular underpinnings of this disease remain elusive but growing evidence suggests that impaired neuron and glial plasticity may be a key underlying mechanism for the trigger of the disorder. Preclinical and clinical studies in this field suggest structural and morphological changes of hippocampus, such as decreased volume and altered mechanisms related with adult hippocampal neurogenesis or dendritic arborization impairments. In particular, several studies refer to alterations in the morphology and numbers of astrocytes, microglia as well as oligodendrocytes both in human patients and in animal models (Polyakova *et al.,* 2015; Rial *et al.*, 2016; Branchi *et al*., 2014).

These observations are supported by functional evidence that includes impairment in the cross-talk among glia and neurons, surrounding changes in the levels of cytokines and neurotransmitters as well as impairment in the synapse and myelination status (Oliveira *et al*, 2016).
Collectively, it is important to study this alternative form of neuro-glial signaling, since may also underlie depressive behavior and then, its manipulation may allow the development of a novel approach for modulation of brain cross-talk and thus restore the healthy phenotype.

1.9. Involvement of glial cells in depressive disorder

Despite broad investigations, the exact neurobiological processes leading to depression are not entirely understood. The most widely accepted classic theory regarding the underlying neuropathology is the monoamine disequilibrium hypothesis, which emphasizes the role of disorganized monoamine neurotransmission in the synaptic cleft (Belmaker and Agam, 2008; Meyer *et al*, 2006). However, it has become evident that this monoamine theory of depression does not explain the wide spectrum of macroscopic and microscopic structural changes that have been frequently documented in the brains of depressed patients. Currently, there are several theories aiming to explain the pathophysiology of depression.

It is increasingly acknowledged that astrocytes play a number of vital roles in the CNS e.g., maintaining synaptic homeostasis, modulating glutamate metabolism, participating in signaling between neurons and glia as well as neurotrophic support. Thus, a theory had been put forward proposing that depressive disorders are the consequence of the disturbed astrocytic functioning (Wang *et al*, 2017).
Many histopathological studies performed on post-mortem brain samples have revealed prominent decreases in astrocyte number in MDD subjects compared to age-matched non-psychiatric controls (Ongur *et al*, 1998; Rajkowska *et al*, 1999). Several studies in literature display a reduced astrocytic population, such as dorsolateral prefrontal (Cotter *et al*, 2002), orbitofrontal (Rajkowska *et al*, 1999) and anterior cingulate cortex (Cotter *et al*, 2001) and amygdala (Altshuler *et al*, 2010; Bowley *et al*, 2002). However, an increase in glial cell density has also been reported in hippocampal regions and dentate gyrus of MDD patients (Stockmeier *et al*, 2004). The increased cell density in the hippocampus indicates a reduction of neuropil per cell, which may contribute to the volume reduction noted in MRI studies in the hippocampus in MDD. This could suggest that alterations in glial density may thus differentially affect specific brain regions. Interestingly, it has been shown that this alteration is age-dependent since that in grey matter of prefrontal cortex of younger depressed

patients (<50 years old), the density of $GFAP^+$ astrocytes is significantly reduced compared to controls of similar age. In contrast, older subjects with late-onset depression showed increased astrocytic population in the same area (Miguel-Hidalgo *et al*, 2000), probably reflecting a mechanism of compensation in the neuronal loss observed in older MDD patients (Rajkowska *et al*, 2005). Alterations of astrocytes have also been reported in the hippocampi of depressed patients. A study focusing on the density of GFAP-positive astrocytes found reduced density of astrocytes in the dentate hilus, but not in other hippocampal subareas (Cobb *et al*, 2016). Furthermore, this decrease was present only in those depressed patients who were not taking antidepressant medications, but not in subjects who were medicated (Cobb *et al*, 2016).

In both humans and animal models, depressive behaviour is 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) as well as are less efficient reactive microglia (Tay *et al*, 2017). Thus, neurons convert lactate to pyruvate which can serve as a substrate for oxidative metabolism (Benarroch, 2005). In addition, astrocytes are involved in glutamate released into the synaptic cleft on adjacent astrocytic processes. In particular, a small amount is taken up by presynaptic neurons for immediate repackaging into vesicles. Astrocytes take up glutamate mainly by Na^+-dependent glutamate transporters (GLAST and GLT1). Interestingly, GLT-1 is an attractive candidate molecule associated with the fundamental processes of MDD since evidences in literature support the reduction of GLT-1 in human post-mortem studies in MDD and thus may be a potential pharmacological target for the treatment of psychiatric diseases (Chen *et al*, 2014; O'Donovan *et al*, 2017). In hippocampus, reduced expression of astrocytes specific glutamate transporter genes (EAAT1, EAAT2) that encodes for GLAST and GLT-1, respectively, was observed (Medina *et al*, 2013; Medina *et al*, 2016).

Furthermore, hippocampus is a region with a high density of microglial cells, especially in the CA1 region, and hippocampal microglial activation demonstrated to be originated by stress and suggested to be implicated in the pathophysiology of MDD, as well as in other psychiatric and stress-related disorders (Walker *et al*, 2013). Microglia are the brain immune cells, responsible for orchestrating the brain innate immune response. They belong to the monocytic-macrophage lineage (Ajami *et al*, 2007) and are therefore natural-born, professional phagocytes (Mallat *et al*, 2005).
Microglia play an essential role during development, phagocytosing the excess of neuroblasts as in the cerebellum (Marin-Teva *et al*, 2004). Instead, during adult life, microglia have been mainly

studied in pathological contest, since microglia became activated or challenged by disease. However, microglia are recognized as the resident brain immune cells and have an important role in the healthy central nervous system. As macrophage in the brain, microglia phagocyte dying cells and cellular debris, without producing inflammation. However, following a pathological insult, like an injury or infection, these cells respond very fast, triggering a reactive response. Activated microglia (i.e. cells that are no longer ramified microglia) change continuously their shape, adopting several altered morphologies, such as hypertrophic, enlarged process or ameboid-like shape (Gemma and Bachstetter, 2013).

1.10. Adult hippocampal neurogenesis and depression

Although the original work of Santiago Ramón y Cajal confirm that a similar process of neuron proliferation in the mature brain was inexistent, evidences on adult neurogenesis remained important in the scientific community. However, the final paradigm changed with evidence from the second half of the 20th century, which confirmed the birth of new methods and techniques that allowed the discovery of adult-born neurons in the brains of rodents (Altman, 1962) and humans (Eriksson *et al*, 1998).

The adult brain contains neurogenic niches in the subventricular zone (SVZ); (Alvarez-Buylla and Lim, 2004; Doetsch *et al*, 1999), which is adjacent to the lateral ventricle, and in the dentate gyrus of the hippocampus (Cameron *et al*, 1993; Kempermann *et al*, 1997; Kuhn *et al*, 1996), where neural stem/progenitor cells (NSPs) continuously generate post-mitotic neurons of different types. The process of adult hippocampal neurogenesis, which start from astrocytic putative neural stem cells (Seri *et al*, 2001) has been divided into six developmental stages: the first one consists of the division of a stem cell, which giving rise to three consecutive stages that it is supposed to be transiently amplifying progenitor cells. These mechanisms may be different by their increasing neuronal differentiation and their proliferative potential. These stages lead finally to the exit from the cell cycle and to a transient post-mitotic stage, during which the selection for long-term survival occurs and network connections are established. Finally, the immature granule cells differentiate in new granule cells (Kempermann *et al*, 2004) (Figure 5).

Adult hippocampal neurogenesis (AHN), is an important mechanism, which can be modulated by stress and antidepressant treatment. AHN is implicated in the behavioural neurobiology of stress-related disorders, especially depression and anxiety (Martin *et al*., 2009). The hippocampus is one of several limbic structures that have been extensively studied in individuals with depression. Magnetic resonance imaging studies have consistently shown a reduction in hippocampal volume (Videbech and Ravnkilde, 2004). Furthermore, it has been suggested a relationship between hippocampal neurogenesis and the hypothalamus–pituitary–adrenal (HPA) axis, which may play a crucial role in the development and in the resolution of depressive symptoms (Schloesser *et al*., 2009).

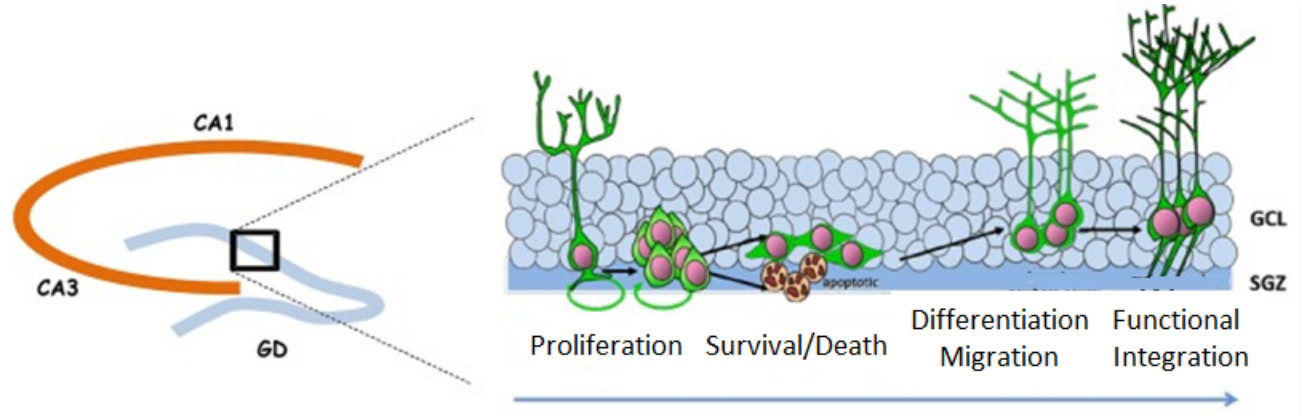

Figure 5. *Schematic representation of adult neurogenesis. New neurons that integrate the granule cell layer (GCL) of the dentate gyrus (DG) originate from stem and precursor cells located in the subgranular zone (SGZ). In the course of their maturation they exhibit specific properties that confers them a unique behavioural function. Modified by Koehl, 2015.*

1.11. Immune System abnormalities and neuroinflammation in depressive disorders

The idea that immune system is involved in a normal neurobehavioral process took hold more than a decade ago, despite the overwhelming evidence demonstrating that inflammatory mediators were involved in the impairment of neurobehavioral plasticity. However, both neurons and glia, through specific receptor may respond to inflammatory cytokines, as well as produce them in healthy conditions, albeit at low levels.

On the contrary, microglial activation and pro-inflammatory cytokines release may have detrimental effects of immune/inflammatory processes involved in neurogenesis, during pathological conditions.

There are a number of investigations which indicate the important relationship between depression and cytokines. This hypothesis is supported by several evidences: treatment of patients with cytokines can produce symptoms of depression; the activation of the immune system is observed in many depressed patients (Valentine, 1998; de Beaurepaire *et al*., 2008). As well as activation of the immune system, and administration of endotoxin (LPS) or interleukin-1 (IL-1) to animals induces sickness behaviour, which resembles depression, and chronic treatment with antidepressants has been shown to inhibit sickness behaviour induced by LPS (Dunn *et al*, 2005).

Pro-inflammatory cytokines can additionally stimulate HPA axis to release glucocorticoids that supress neurogenesis (Liu *et al*, 2003). Several research groups have provided a direct link between IL-1β and neurogenesis, involving at least two mechanisms. The first one is explained by a direct effect of IL-1 on neuronal progenitors in the sub-granular zone of dentate gyrus, through their receptor, IL-1R1, expressed in these cells. In particular, in the adult hippocampal progenitors, exposure to IL-1β leads to a decreased percent of proliferating progenitors *in vitro* (Koo and Duman, 2008). The second mechanism is indirect and includes IL-1-induced glucocorticoids secretion (Goshen *et al*, 2008), particularly important in stress-induced neurogenesis mechanism.

At the same time, the effects of TNFα in neurogenesis was assessed by several approaches. In particular it seems that this cytokine has a direct effect on neural precursor cell proliferation, including a marked suppression of neurogenesis (Ben-Hur *et al*, 2003; Monje *et al*, 2003). Even if in another study, *in vitro* TNFα did not produce any effect, but increased the percentage of astrocytes and reduced the number of neurons, whether applied during differentiation of precursor (Kohman and Rhodes, 2013).

Some characteristics shown by a subpopulation of depressed patients are immune dysregulation and chronic inflammation (Rook and Lowry, 2008; Schiepers *et al*, 2005). The cytokine hypothesis assumes that in depression the pro-inflammatory cytokines, including IL-6, IL-1β and TNF-α,

increase while the anti-inflammatory cytokines, including interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), decrease, making the immune response tend to inflammation.

This unbalance, with an increase of pro-inflammatory cytokines leads to an inhibition of the negative feedback of the HPA axis with concomitant increase the permeability of the blood–brain barrier. This mechanism reduces the synthesis of 5-HT, disturbs the glutamatergic systems, and results in depression (Maes, 2011; O'Brien *et al*, 2004; Schiepers *et al*, 2005). The early theories mainly focus on peripheral inflammation, but new theories, such as the neuroinflammation hypothesis pay more attention to central inflammation (Leonard, 2018; Singhal and Baune, 2017). The neuroinflammation hypothesis emphasizes the adverse effects on the CNS exerted by excessive pro-inflammatory cytokines released by microglia, which can be induced by various factors such as psychological stress, disease, and infection (Singhal *et al*, 2017). Glia cells are important modulators of neuroimmune response. Although different theories focus on different aspects, all of them agree on the neuroinflammation impairment induced by neuroglia dysfunction results in depression. Chronic inflammation is often associated with the development of symptoms in depressed patients (Benton *et al*, 2007; Goldberg, 2010). Indeed, activation of the peripheral immune system leads to increased cytokine levels that are actively transported into the CNS. In particular, this leads to a higher stimulation of astrocytes and microglial cells, which in turn produce cytokines via feedback mechanism (Muller and Ackenheil, 1998). Although the intracellular mechanism is not completely understood, seems that microglia, besides releasing inflammatory mediators as well as cytokines and glutamate, is able to metabolize kynurenine transported to the CNS into quinolinic acid, a neurotoxic compound (Dantzer *et al*, 2008). Astrocytes seem not to be able to uptake the excess of glutamate that together with quinolinic acid will enhance glutamatergic neurotransmission leading to the development of symptoms of depression.

Furthermore, in the hippocampus a number of studies have shown IL1-β to produce decreased in GluA1-AMPA R subunit expression and increased alpha5-GABA-A R subunit expression (Lai *et al*. 2006; Wang *et al*., 2012). In conclusion, these novel findings provide another plausible molecular mechanism underlying inflammation-induced memory deficit illustrating the importance of immune–neuronal interactions in synaptic plasticity.

1.12. Microbiota–gut–brain axis dysfunction in depressive disorders

The gut of mammals is also called “gut brain” because it has its own nervous system (enteric nervous system) and can make relatively independent responses to external signals (Lima-Ojeda *et al*, 2017). Our gut harbours trillion of symbiotic microorganisms are important and beneficial not only for the regulation of host physiology, but also for suitable development of CNS and brain responses (Cryan and O'Mahony, 2011; Dinan and Cryan, 2013; Qin *et al*, 2010).

The microbiota-gut-brain axis is a bidirectional pathway, i.e. the brain modulates the activity of the gut and *vice versa*, ensuring homeostasis of the host system (De Vadder *et al*, 2014; Sherwin *et al*, 2018). The gut brain is constituted by microbial organ, 90–95% of the total cells of which are microorganisms including bacteria, archaea, fungi, viruses, and some protozoa, and the metabolism, immune system, and signal transmission are all closely related with microbiota. Depressed patients often have gut brain dysfunction, such as appetite disturbances, metabolic disturbances, functional gastrointestinal disorders, and gut microbiota abnormalities (Collins and Bercik, 2009; Evrensel and Ceylan, 2015).

Depression is not just a simple psychiatric disease or mental disorder, but also a systemic disease. Depressed patients often suffer from various disorders simultaneously, such as brain dysfunction and periphery dysfunction, such as HPA axis disturbances, immune dysregulation, and gut brain disturbances that interplay with each other. 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).

The gut microbiota hypothesis assumes that depression is closely related with gut microbiota, and microbiota–gut–brain axis dysfunction is the main pathological basis of this condition. Gut microbiota alterations are a direct stimulus and key risk factor concealing in environmental and genetic risk factors, as well as microbiota regulation is the promising method for depression therapy and prevention. An increasing amount of research, exploring the gut brain in the last decades supports the hypothesis from different aspects (Kennedy *et al*, 2017; Rieder *et al*, 2017).

Clinic studies have presented that the gut microbiota of depressed patients is significantly different from that of healthy controls. Some research found that both the microbiota diversity and richness declined in patients (Jiang *et al*, 2015; Naseribafrouei *et al*, 2014). Indeed, microbiota composition is altered not only in patients, but also in animal models with this symptomatology (Hoban *et al*, 2016; Kelly *et al*, 2016). Stress disturbs the microbiota and increases the susceptibility of depression.

Stressful life events are important inducements of depressive disorders, and they are often used in animal depression research. Chronic stress not only impacts the mind and the stress response system, but also disturbs the gut microbiota (Holdeman *et al*, 1976; Marin *et al*, 2017).

Microbiota dysbiosis can induce various physiological and psychological diseases, and microbiota restoration brings improvement to these diseases (Kennedy *et al*, 2017; Rieder *et al*, 2017).

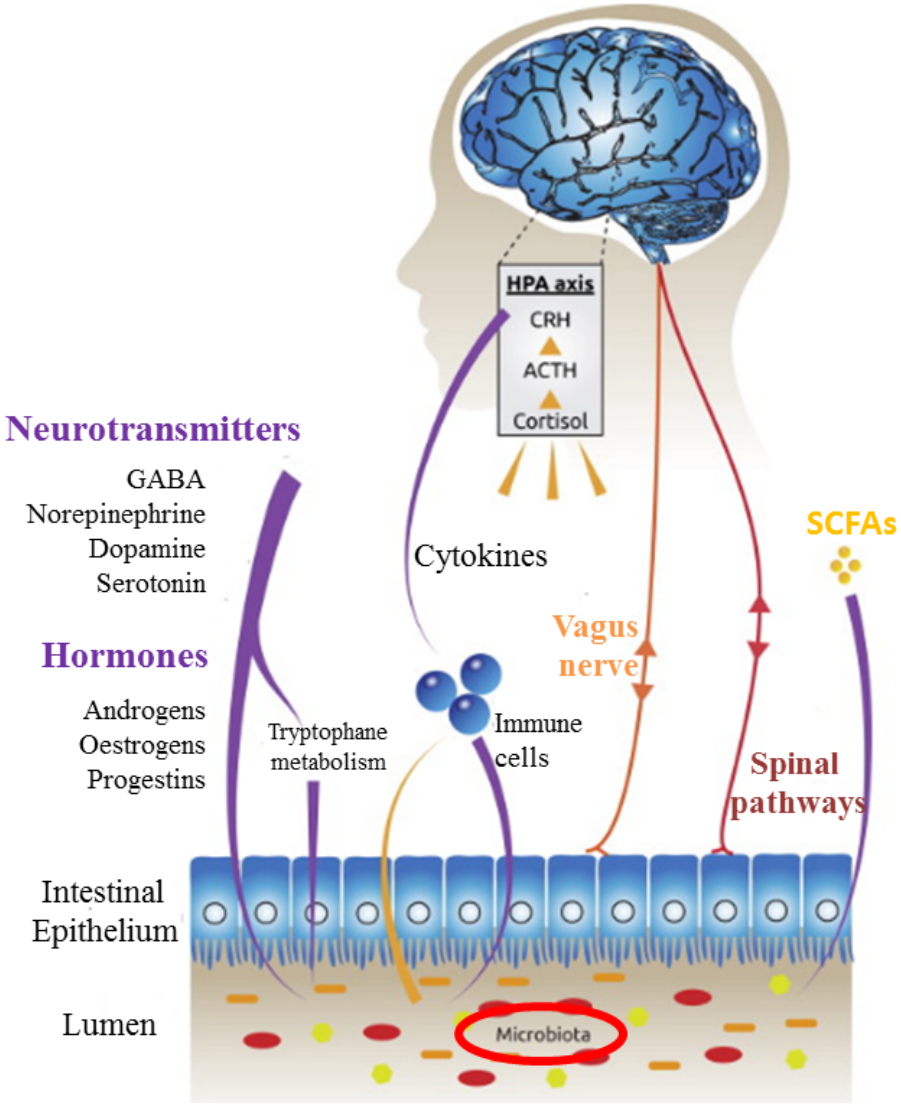

Figure 6. *The gut microbiome-brain axis. The gut microbiome consists by the microbiota, their genomes and their products can influence brain function through several mechanisms, including the production of short chain fatty acids (SCFAs) and neurotransmitters, the modulation of the release of cytokines by immune cells, triptophane metabolism and the vagus nerve. On the other hand, the brain can influence the gut microbiota via regulation of endocrine systems such as hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal axes. Figure modified by Dinan et al., 2015.*

2. AIMS

Finasteride is a reversible inhibitor of the enzyme 5α-R, which converts neuroactive steroids (i.e., important physiological regulators of the nervous system), such as progesterone and testosterone into their active metabolites. By inhibiting 5α-R activity, finasteride reduces DHT levels in prostate and in hair follicles and improve the conditions of human benign prostatic hyperplasia and androgenic alopecia (AGA) (Traish *et al*, 2015). Despite of the wide therapeutic use of this inhibitor in these pathological conditions, its effects in the central nervous system have been poorly explored.

That is particularly important, because a subset of men taking finasteride for AGA show, not only during the treatment, but also after discontinuation, a wide symptomatology including depression, erectile dysfunction, endocrine alterations and musculoskeletal manifestations (Giatti *et al*, 2018a).

Moreover, as self-reported by the patients (Altomare *et al*, 2002; Irwig, 2012b; Rahimi-Ardabili *et al*, 2006; Traish *et al*, 2015) and as recently ascertained by two different clinical studies, these important side effects, like for instance depression, may persist even after discontinuation of the treatment (Basaria *et al*, 2016; Melcangi *et al*, 2017). Thus, these patients are affected by post-finasteride syndrome (PFS).

Like others psychiatric disorders PFS is characterized by persistent alteration of plasma neuroactive steroid levels, which may be associated with major depression. In particular, 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*, 2015b; Melcangi *et al*, 2013a; Melcangi *et al*, 2017).

This effect was also observed in plasma and CSF of male rats treated with finasteride for 20 days and evaluated after one month of drug suspension. Further observations confirmed persistent alterations in neuroactive steroid levels also in the rat brain (Giatti *et al*, 2016b).

Furthermore, as reported in humans and in animal models, depression is characterized by increased inflammation and gliosis, decreased neurogenesis and neuron survival and mitochondrial impairment (Blier, 2013; Masi *et al*, 2011). Moreover, data in literature 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).

On this basis, in the present study we have explored: (1), whether subchronic finasteride treatment induces depressive-like behavior in male rats; (2), whether this alteration persists by one 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 inflammatory parameters in plasma as well as with changes in gut microbiota.

3. METHODS

3.1. 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 one month after withdrawal (i.e. one month after the last injection; n=20), respectively. In each experimental group, the rats were randomly assigned to vehicle-treated rat group (Control) and to finasteride-treated group (Finasteride). The vehicle and drug solutions were administrated daily for 20 days.

3.2. Behavioral test: Forced swim test

Forced swim test is a classic paradigm of despair which measures depressive behaviour in rodents. Animals were introduced for 6 minutes *per* day for two consecutive days (i.e., pre-test session on day 1 and test session on day 2) into a methacrylate cylinder, a transparent plastic resistant material, containing water at 23-24°C (Figure 7). The water temperature is very important: a lower temperature causes hyperactivity that could stress the animal, while, on the contrary, a higher temperature could relax the animal (Jefferys and Funder, 1994). It was analyzed only the time of immobility, which is considered a measure of depressive-like behavior in both test days.

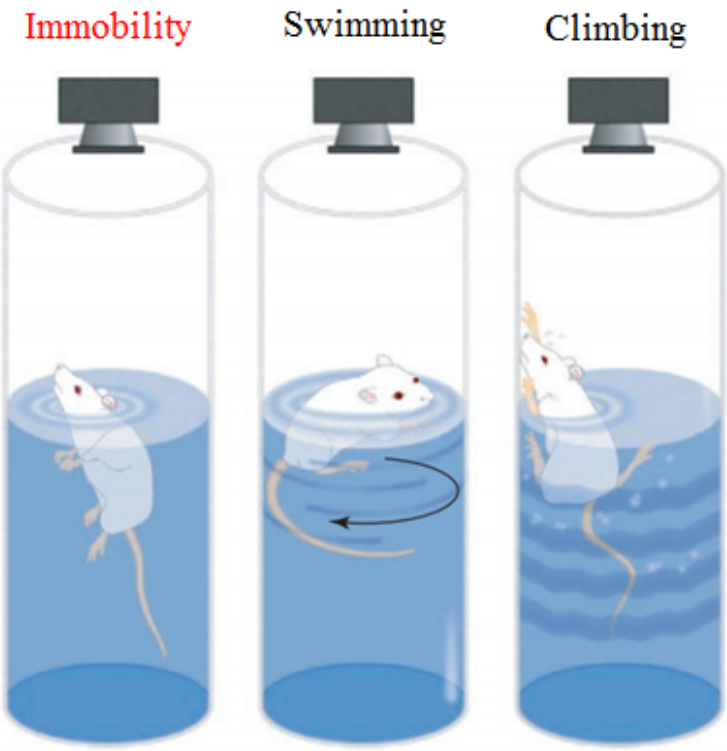

Figure 7. Cartoon of rats undergoing forced swim test (FST) behaviours. At least three different forms of behaviour (i.e., swimming, climbing and immobility) may be observed. In our study immobility was considered. Figure modified by Cryan *et al*, 2002.

3.3. 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 bw, as published elsewhere (Llorens-Martin *et al*, 2010). Both experimental groups received one injection of BrdU 24 h before sacrifice.

3.4. Immunohistochemistry (IHC)

Rats were deeply anesthetized and then perfused through the left cardiac ventricle first with pre-warmed (37ºC) 0.9% NaCl, followed by 4% paraformaldehyde (pH 7.4; 37ºC). The brains were dissected out and post-fixed overnight at 4ºC 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 3 (pH3) is a marker for cells undergoing mitosis. We performed double IHC for pH3 and BrdU to analyze cell division. For pH3/BrdU double IHC staining, sections were pre-incubated for 25 minutes with 2N HCl at RT to ensure antigen unmasking. Then, sections were washed 5 times for 5 minutes in 0.1 M PB containing 1% Triton X-100 and 1% bovine serum albumin. Double IHC 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 4ºC. 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 minutes 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 minutes 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 PB. 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.

3.5. 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 dissector method in the hilus of the dentate gyrus of the hippocampus using total section thickness for dissector 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. In addition, the percentage of Iba1 immunoreactive cells with different morphologies was also assessed. Cells were classified in five morphological types: type I, cells with few cellular processes (two or less); type II, cells showing three to five short branches; type III, cells with numerous (>5) and longer cell processes and a small cell body; type IV, cells with large somas and retracted and thicker processes and type V, cells with amoeboid cell body, numerous short processes and intense Iba-1 immunostaining (Diz-Chaves *et al*, 2012). Successively, cells were grouped in two morphological types, resting and reactive, type I-II and type III-IV-V, respectively.
For each animal, a range of 450-600 cells were analysed in the hilus of the dentate gyrus of the hippocampus.

3.6. 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 1024x1024 pixels. Granule cell density was performed counting each Z-stack in Image-J program.

3.7. Real time polymerase chain reaction (RT-PCR)

Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) mRNA levels were assessed in the hippocampus by real-time PCR. RNA from frozen tissue was extracted using Directzol™ 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 Laboratories, 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′-GGGATTTTGTCGTTGCTTGTC-3′; for TNF-α, forward, 5′-CTTCTCATTCCTGCTCGTGG-3′ and reverse, 5′-TGATCTGAGTGTGAGGGTCTG-3′; for 36B4, forward, 5′-GGATGACTACCCAAAATGCTTC-3′ and reverse, 5′-TGGTGTTCTTGCCCATCAG -3′. All genes were normalized to 36B4.

3.8. 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 (StratecMolecular, 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.

3.9. Plasma Preparation

Trunk blood was collected at the moment of sacrifice and collected in EDTA-treated tubes. Cells are removed from plasma by centrifugation for 15 minutes at 3000 rpm using a refrigerated centrifuge. The resulting supernatant (plasma) was stored at -20°C, until ELISA analysis to detect [IL-1β] plasma in finasteride treated-rats vs control rats.

3.10. ELISA IL-1β

The first step in an ELISA experiment is the immobilization of the antigen in a sample to the wall of the wells of a microtiter plate. This can be achieved by direct adsorption to the plate's surface or by using a "capture antibody". The capture antibody has to be specific to the target antigen and is mainly used in a specific ELISA type called "sandwich ELISA". After immobilization, a detection antibody is added, which binds to the adsorbed antigen thereby leading to the formation of an antigen-antibody complex. The detection antibody is either directly conjugated to an enzyme, such as horseradish peroxidase (HRP), or provides a binding site for a labeled secondary antibody. The assay used in this experiment employs the quantitative sandwich enzyme immunoassay technique, following protocol of kit (R&D System #RLB00). Briefly, a polyclonal antibody specific for rat IL-1β was pre-coated into a microplate. Standards, Control, and samples were pipetted into the wells and any rat IL-1β present is bound by the immobilized antibody. After washing away any unbound

substances, an enzyme-linked polyclonal antibody specific for rat IL-1β was added to the wells. Following a wash to remove any unbound antibody enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of rat IL-1β bound in the initial step. Then, the sample values were read off the standard curve. The duplicate readings for each standard, control, and sample were averaged and subtracted to the average zero standard optical density (O.D.).

3.11. 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 behavioural analysis (n=10 *per* experimental group) and RT-PCR analysis (n=6 *per* experimental group) were analysed by two-way analysis of variance (ANOVA), with treatment and time as two independent variables, followed by the Bonferroni post hoc test; ELISA analysis was analysed by Student's t test (n=5 *per* experimental group); number of immunoreactive cells (n=4 *per* experimental group) were analysed with the Mann-Whitney non-parametric test. For microbiota 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 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).

4. RESULTS

4.1. Behavioural analysis: Forced swim test

Previous studies performed by our research group have shown that a subchronic treatment in male finasteride treated-rats for 20 days, leads to alteration in the levels of neuroactive steroids in plasma and in CSF. This occurs both after treatment and after discontinuation of the drug (i.e., one month) (Giatti *et al*, 2016b). Moreover, these observations confirmed persistent alterations in neuroactive steroid levels also in the brain (Giatti *et al*, 2016b). It is interesting to note that, even if finasteride is a blocker of 5a-R, its treatment or withdrawal did not only affect this enzyme, and consequently the production of 5alpha-reduced metabolites of PROG and T, but also the formation of other neuroactive steroids (Giatti *et al*, 2016b).

In addition, not only the levels of neuroactive steroids but also their receptors are affected. Indeed, classical steroid receptors as well as non-classical steroid receptors, like for instance different GABA-A receptor subunits, were modified by the finasteride treatment or its withdrawal (Giatti *et al*, 2016b)

Alterations in the levels and signalling of neuroactive steroids in animal model is important, because likewise to other psychiatric disorders, PFS is characterized by persistent alteration of neuroactive steroid levels, which may be associated with major depression. In particular, 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 T and PROG, but also to a global alteration of neuroactive steroid levels (Caruso *et al*, 2015b; Melcangi *et al*, 2013b; Melcangi *et al*, 2017). Moreover, as self-reported by the patients (Altomare *et al*, 2002; Irwig, 2012b; Rahimi-Ardabili *et al*, 2006; Traish *et al*, 2015) and as recently ascertained by two different clinical studies depressive symptomatology may persist even after discontinuation of the treatment (Basaria *et al*, 2016; Melcangi *et al*, 2017).

On this basis, in the present study we have explored whether subchronic finasteride treatment induces depressive-like behavior in male rats and whether this alteration persists by one month after the end of the treatment. Thus, we have investigated the possible behavioral consequences of the modifications in neuroactive steroid levels caused by finasteride treatment and its withdrawal. In particular, we have assessed depressive-like behavior in rodents with the Porsolt forced swim test (FST) (Castagne *et al*, 2011).

The animals were introduced for 6 minutes per days in two consecutive days (i.e. pre-test session day 1 and test session day 2) into a cylinder containing water. Then, we have measured the time of immobility as an index of depressive-like behaviour.

The results of FST include the effects of finasteride in pre-test session and in the test session in order to differentiate the possible effects of finasteride in the two different days of test sessions.
Then, this analysis was also repeated for withdrawal animals.

In the figure 8, the results of FST shown between animals treated with vehicle or finasteride in pre-test session vs test session, at the end of the treatment. No significant differences were detected in the time of immobility between animals treated with vehicle vs finasteride.

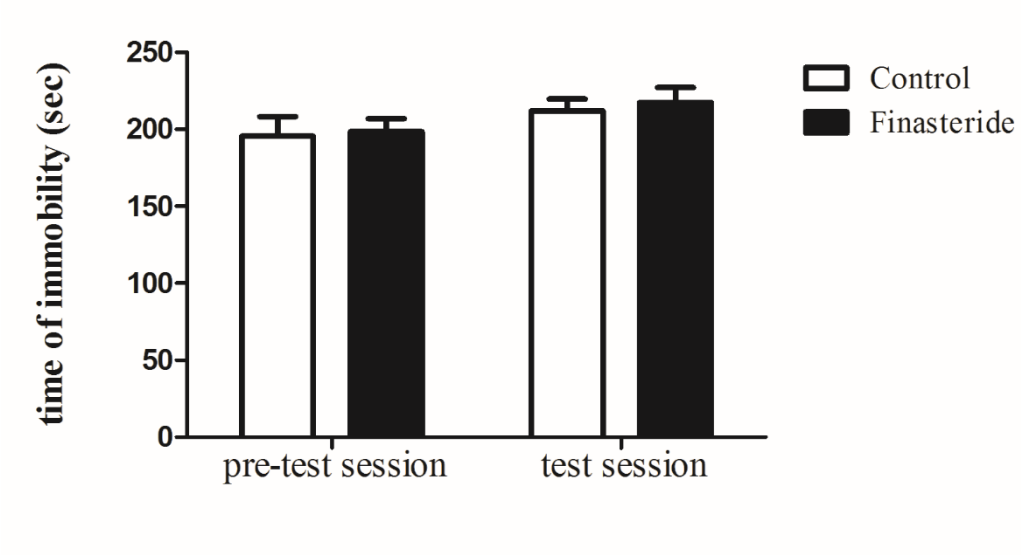

Figure 8. *Effect of finasteride on depressive-like behaviour. Data represents the immobility time in the forced swim test in animals treated with vehicle (control) or finasteride in pre-test session vs test session, at the end of the treatment. Data are represented as mean ± SEM, n=10 for each experimental group.*

However, the results of FST in pre-test session vs test session at withdrawal have shown that between animals treated with vehicle or finasteride there is no interaction, but a significant effect of treatment and time (Figure 9). Moreover, Bonferroni post hoc test show that there is a significant effect at the test session.

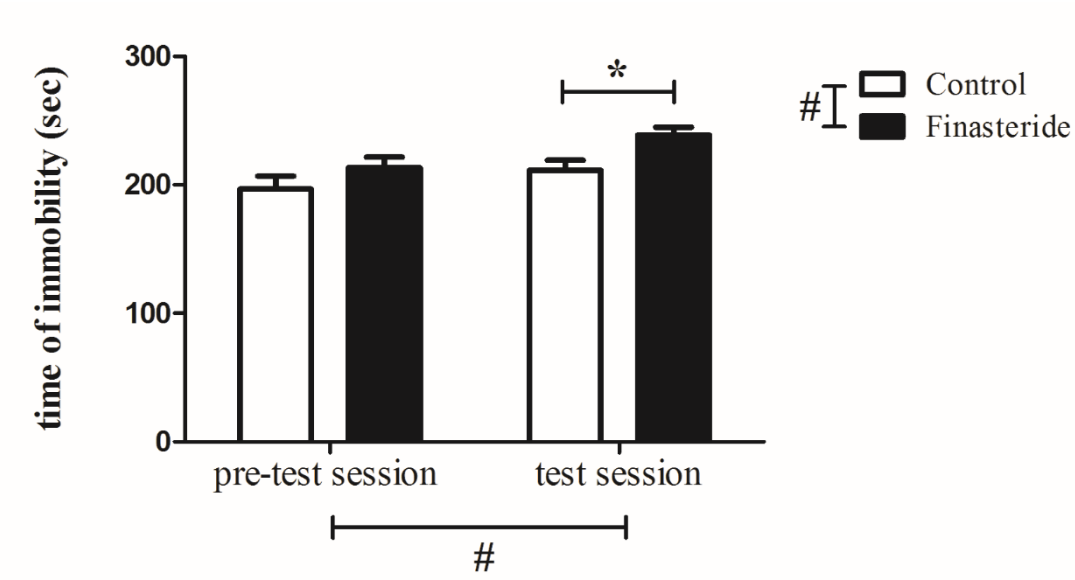

Figure 9. *Effect of finasteride on depressive-like behaviour. Data represents the immobility time in the forced swim test in animals treated with vehicle (control) or finasteride in pre-test session vs test session, at withdrawal. Data are represented as mean ± SEM, n=10 for each experimental group. The effect of treatment, time and the interaction treatment by time were analysed using two-way ANOVA (significance: # $p < 0.05$) followed by Bonferroni post hoc test (significance: * $p < 0.05$).*

Furthermore, we analyse whether differences may occur only at test section (day 2), between the two different experimental groups (i.e. after treatment and after withdrawal). Two-way ANOVA revealed a significant effect of treatment (F= 4.22, p = 0.0474) and no interaction as well as no effect of time (Fig. 10). Bonferroni post hoc test indicates that there is a significant effect at withdrawal (t= 2.427, p <0.05). Thus, one month after withdrawal animals treated with finasteride showed a significantly

higher immobility in the Porsolt test compared with animals treated with vehicle during the test session (day 2).

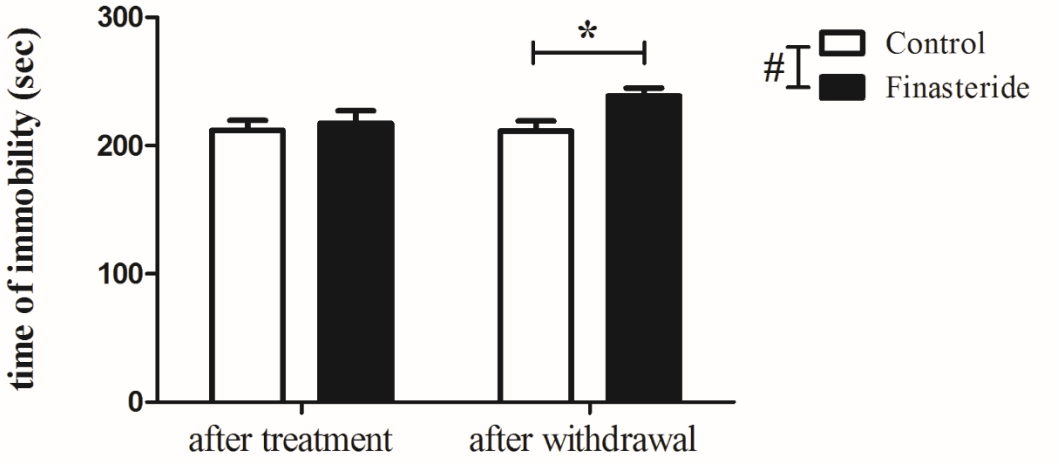

***Figure 10**. Effect of finasteride on depressive-like behaviour. Data represents the immobility time in the forced swim test in animals treated with vehicle (control) or finasteride, after treatment vs after withdrawal, at test session (day 2). Data are represented as mean ± SEM, n=10 for each experimental group. The effect of treatment, time and the interaction treatment by time were analysed using two-way ANOVA (significance: # $p < 0.05$) followed by Bonferroni post hoc test (significance: * $p < 0.05$).*

4.2. Neurogenesis analysis

Changes in hippocampal neurogenesis in rodents have been associated with modifications in depressive-like behaviors (Santarelli *et al*, 2003). Moreover, as reported in humans and in animal models, depression is characterized by decreased neurogenesis and neuron survival (Blier, 2013; Masi *et al*, 2011). Thus, decreased neurogenesis in the dentate gyrus is linked to increased immobility in the forced swim test (Snyder *et al*, 2011).

On the basis of these observations and on the finding that in our experimental model depressive-like behavior was observed we have ascertained whether modifications in adult hippocampal neurogenesis was also present.
In particular, we have analyzed three important processes involved in neurogenesis, such as the cell proliferation (pH3 staining), the cell survival (BrdU staining) and granule cell density (DAPI staining) in the granule cell layer of dentate gyrus of hippocampus.

By immunohistochemistry analysis we have found no significant differences 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 (C:21±9.95, F:7.5±5.68) nor one month after withdrawal (C:6±2.45, F:0±0).
However, at the end of the treatment the number of pH3 immunoreactive cells was significantly increased ($p = 0.0294$) in finasteride-treated rats compared to vehicle injected animals (Figure 11D). On the contrary, the number of pH3 immunoreactive cells was significantly decreased ($p = 0.0284$) one month after finasteride withdrawal compared to vehicle injected animals (Figure 11D). Granule cell density in the granular cell layer was unmodified at the end of treatment but significantly decreased after one month of finasteride withdrawal ($p = 0.0286$) (Figure 11E).

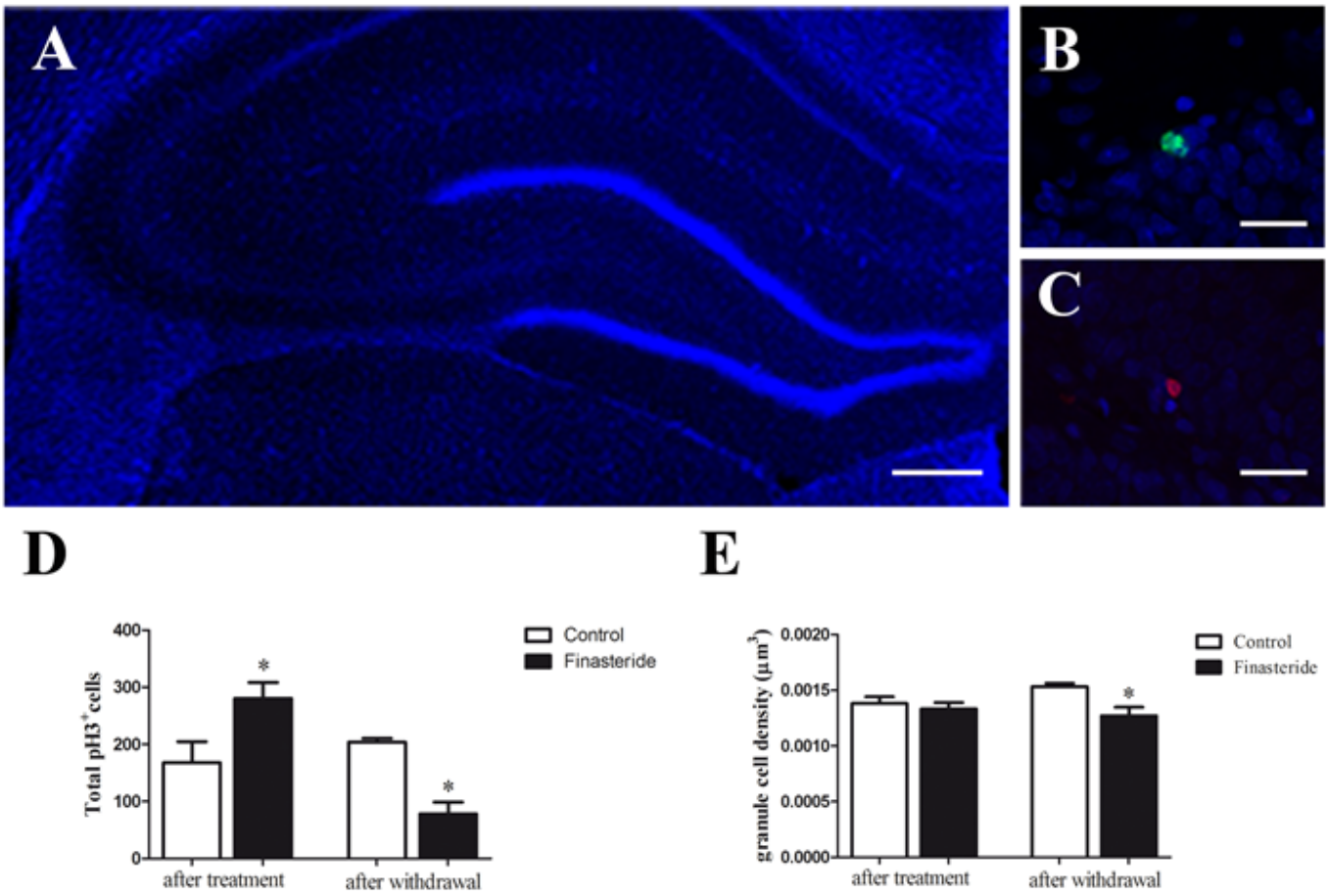

Figure 11. *Effects of finasteride on adult hippocampal neurogenesis.* ***A****) Representative DAPI staining of the dentate gyrus. Scale bar, 400 µm.* ***B****) Example of a pH3 immunoreactive cell (green) by confocal microscopy in the subgranular layer of the dentate gyrus. The section was counterstained 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.*

4.3. Morphometrical Analysis of Glia

In both humans and animal models, depressive behaviours are associated to an increased neuroinflammation and reactive gliosis (Yirmiya *et al*, 2015). This is in agreement, with the finding that microglia and astrocytes are the main mediators of the inflammatory response.

On this basis, we have explored whether finasteride treatment and/or withdrawal was associated with modifications in the morphology and/or in the density of glial cells. To this aim, GFAP and Iba-1 immunoreactive cells were evaluated in our experimental groups.

As reported in figure 12B, finasteride treatment was able to increase the number of GFAP immunoreactive astrocytes in the hilus of the dentate gyrus compared to vehicle-injected animals. Interestingly, this effect was significantly 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 (Figure 12B).

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, neither at the end of the treatment (C: 5339±138.3, F:5366±428.7) nor one month after withdrawal (C: 4665±264.1, F: 4318±198). However, at the end of the treatment, animals injected with finasteride had a higher proportion of Iba-1 immunoreactive cells with a reactive phenotype than the animals injected with vehicle (Figure 13A-B).

One month after the end of the treatment, most Iba-1 immunoreactive cells showed a resting phenotype in both vehicle and finasteride injected animals (Figure 13C-D).

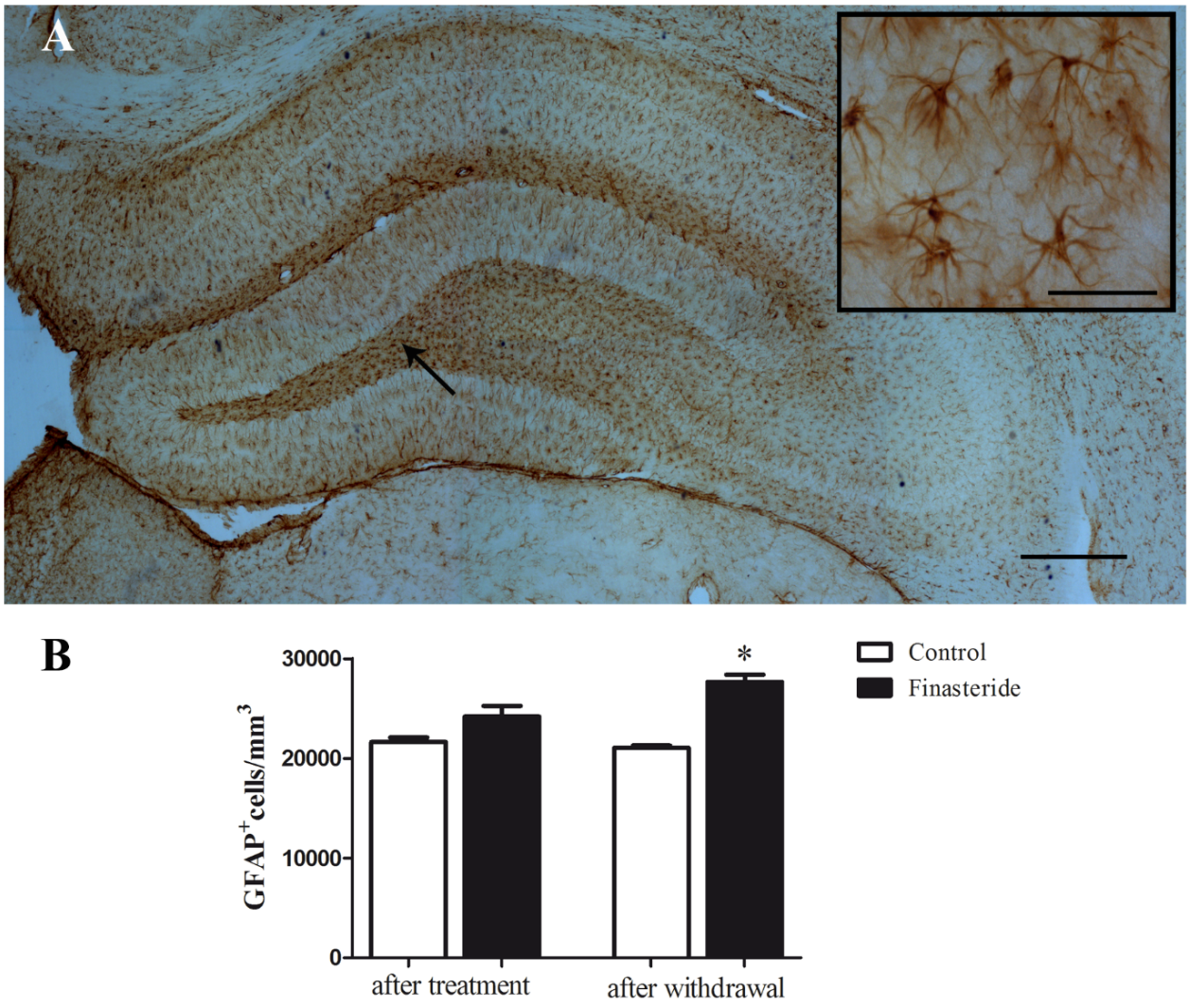

***Figure 12**. Effect of finasteride on the density of GFAP immunoreactive astrocytes in the hilus of the dentate gyrus. **A)** Representative image of the dentate gyrus showing immunoreactivity for GFAP in the hilus (arrow). Picture scale bar, 90 µm. The inset shows GFAP immunoreactive astrocytes in the hilus at higher magnification. Scale bar, 30 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.*

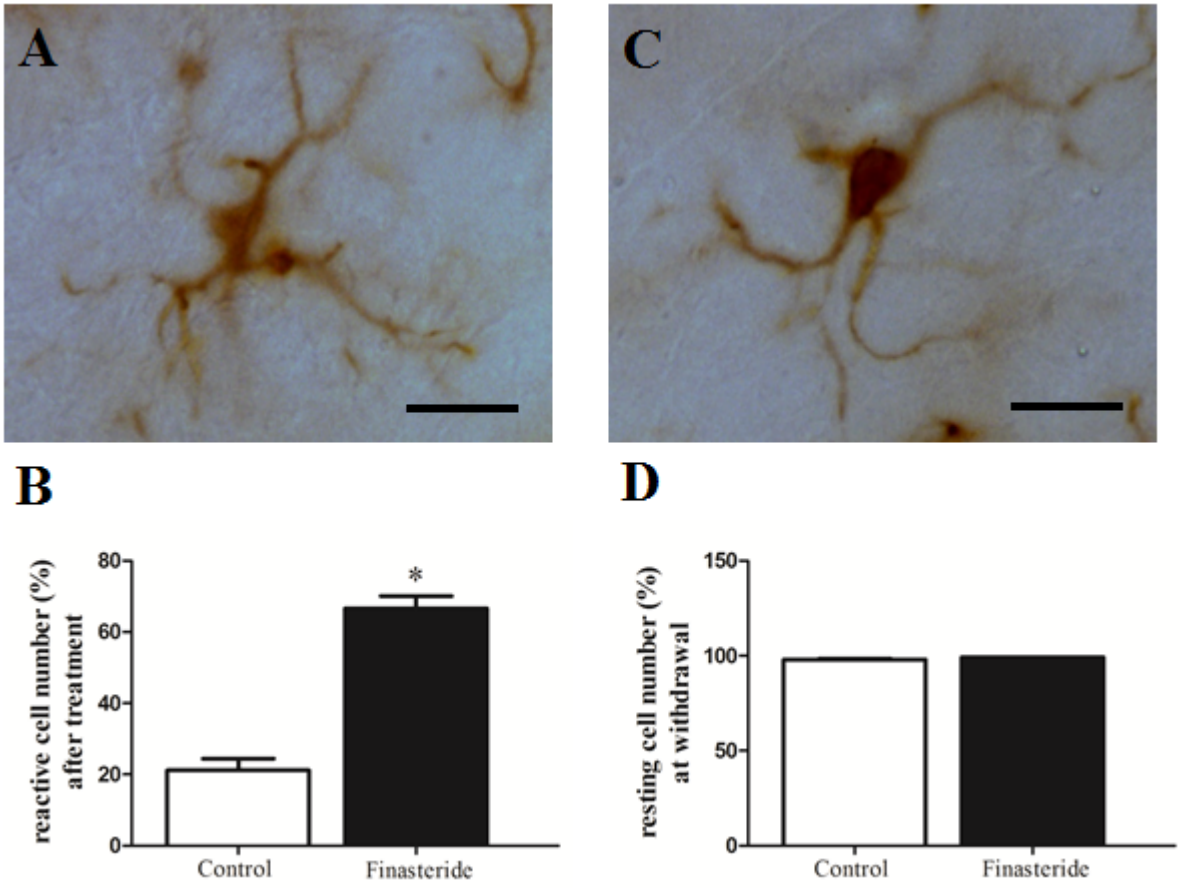

Figure 13. *Morphological classification of Iba1-positive cells. Examples of Iba-1 immunoreactive cells with reactive (**A**) and nonreactive (**C**) phenotypes. Scale bars represent 12 um. **B**) Number (%) of Iba1-positive cells with reactive phenotype in the hilus of the dentate gyrus in in animals treated with vehicle (control) or finasteride at the end of the treatment. **D**) Iba1-positive cells (%) with resting phenotype in the hilus of the dentate gyrus in animals treated with vehicle (control) or finasteride at one month after withdrawal. Data are expressed as mean ± SEM, n=4 for each experimental group. *, significant difference, $p < 0.05$, Mann-Whitney non-parametric test.*

4.4. Neuroinflammation assessment

Reactive gliosis is associated with increased levels of pro-inflammatory molecules in the brain (Hostenbach *et al*, 2014; Wang *et al*, 2018). In agreement with this concept, the effect of finasteride on the number of GFAP immunoreactive astrocytes reported above (Figure 12B) was associated with changes in the mRNA levels of the pro-inflammatory cytokines. Indeed, as reported in Figure 13, 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. 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, by two-way ANOVA analysis, the mRNA levels of IL-1β in the hippocampus were not significantly different (Figure 14). However, it is important to note that, analysing 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).

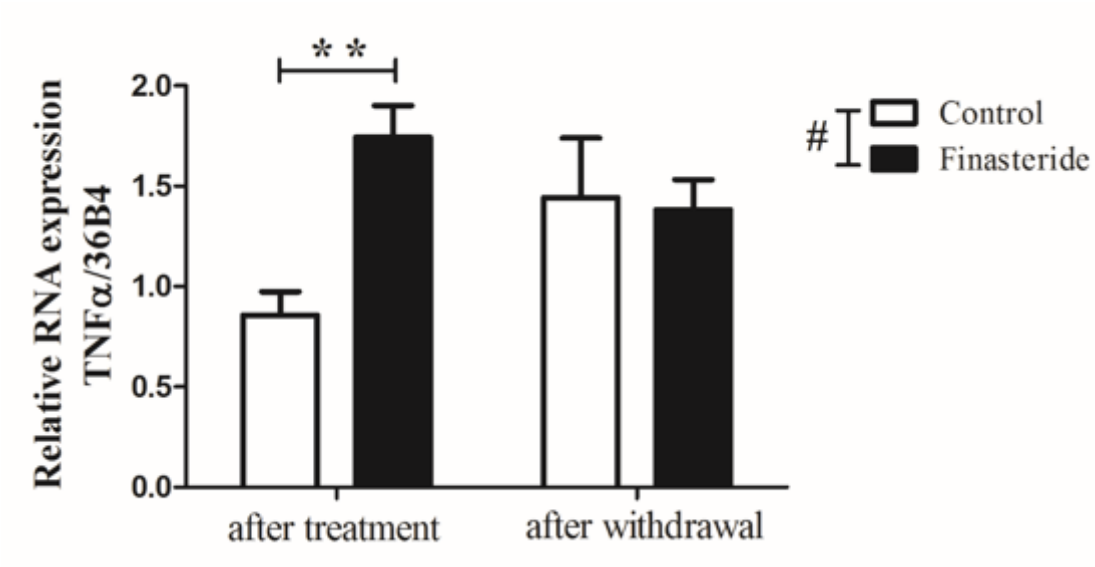

Figure 14. *Effect of finasteride on the mRNA levels of TNFα in the hippocampus. TNFα mRNA levels in animals treated with vehicle (control) or finasteride at the end of the treatment and at one month after withdrawal. Data are expressed as mean ± SEM, n=6 for each experimental group. The effect of treatment, time and the interaction treatment by time were analyzed using two-way ANOVA (significance: # $p < 0.05$) followed by Bonferroni post hoc test (significance: ** $p < 0.01$).*

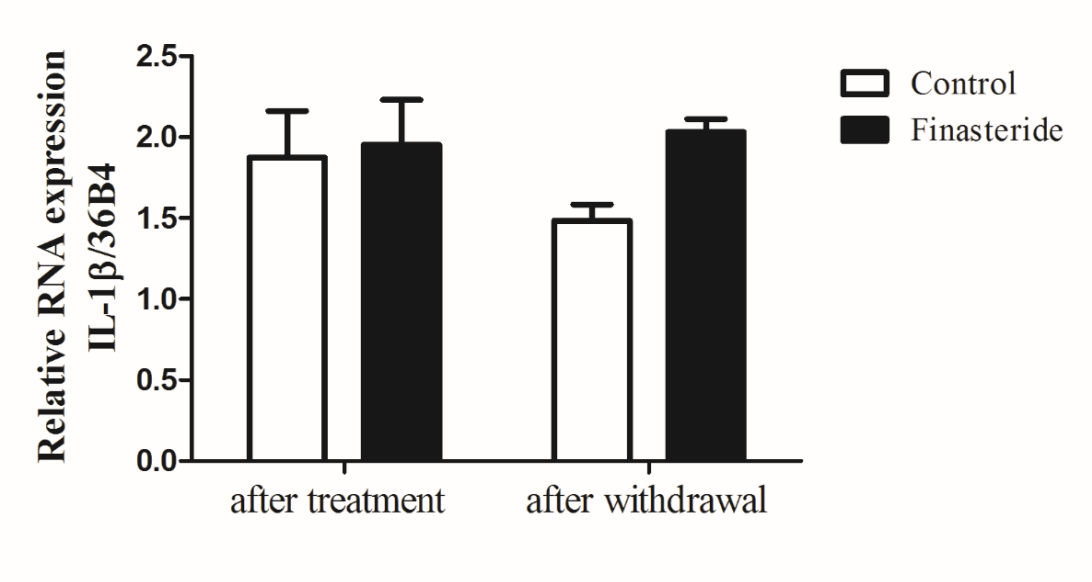

Figure 15. *Effect of finasteride on the mRNA levels of IL-1β in the hippocampus. IL-1β mRNA levels in animals treated with vehicle (control) or finasteride at the end of the treatment and at one month after withdrawal. Data are expressed as mean ± SEM, n=6 for each experimental group.*

4.5. Peripheral inflammation assessment

As reported in literature, a possible involvement of inflammation has been also related to depression. For instance, a meta-analysis performed by Dowlati and collaborators (Dowlati *et al*, 2010) on 24 studies, showed significant elevated levels of IL-6 and TNF-α in patients with major depression when compared with control patients. In addition, Smith suggested that depression was associated with increased secretion of cytokines (especially IL-1) by macrophages (Smith, 1991). Several studies have focused on the possible elevation of cytokines in depressed patients (see review Dunn et al., 2005). For instance, IL-1β, IL-6 and the IFN's have been reported to be increased in the plasma of depressed patients (Maes *et al*, 1993a; Maes *et al*, 1993b; Maes *et al*, 1994).

On this basis, in our animal model, whether finasteride treatment may affect pro-inflammatory cytokines levels also in plasma (i.e. in the peripheral body) was investigated. Thus, an ELISA test was used to evaluate the plasma levels of rat interleukin-1 beta (IL-1β), as example of pro-inflammatory cytokine.

Data so far obtained, indicate a significant increase in plasma levels of IL-1β in finasteride-treated vs control animals at withdrawal period (Control: 223.8±1.79 pg/ml vs Finasteride: 234.3±3.73 pg/ml; n=5; p=0.0349, Student's t-test). Thus, when in our model the depressive-like behavior was observed. On the contrary, after drug treatment, a significant decrease in plasma levels of IL-1β in finasteride-treated vs control animals was observed (Control: 249±1.64 pg/ml vs Finasteride: 231±2 pg/ml; n=5; p=0.0001, Student's t-test).

4.6. Gut Microbiota Analysis

Literature data suggest a link between gut microbiota and development and/or manifestation of neuropsychiatric disorders, such as depression and anxiety (Borgo *et al*, 2017; Foster *et al*, 2013). To this aim, we have explored whether possible changes induced by finasteride may affect the gut microbiota composition.

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 24h after 20-days treatment.

Data obtained show for the first time that a subchronic treatment with finasteride was able to affect gut microbiota of male rats. In particular, we did not observe a statistically significant change in α-diversity assessed by Chao1 (p=0.36) and Shannon indices (p=0.14), but β-diversity analysis, revealed a significant diversity based on weighted (p=0.03) and unweighted UniFrac distances (p=0.04) (Figure 16-17). At phylum level (Figure 18), 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 (Figure 18). 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 (Figure 18). 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 (Figure 18).

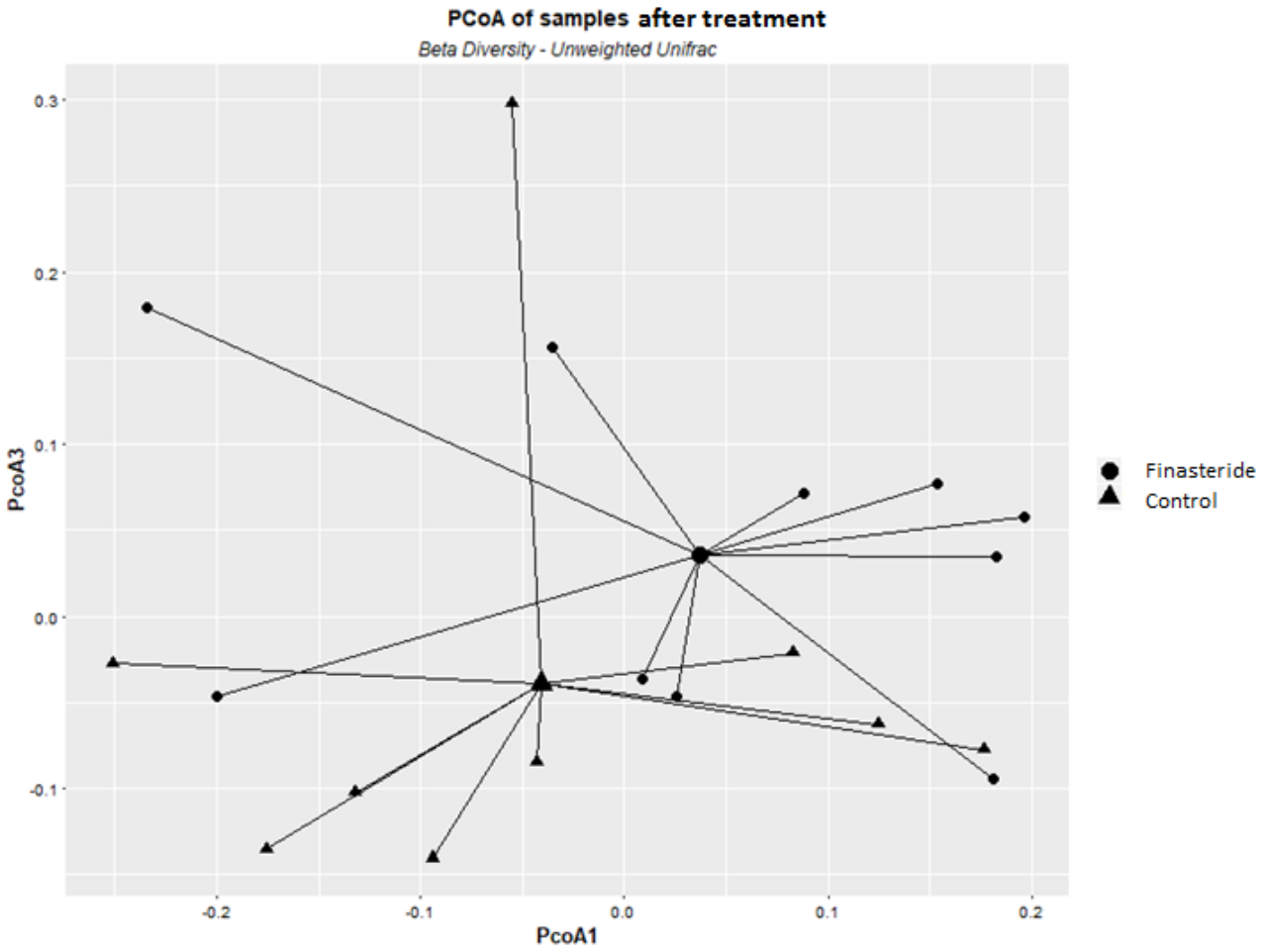

Figure 16. *Gut microbiota modulation at the end of finasteride treatment. Principal Coordinate Analysis (PCoA, beta-diversity) plot according to unweighted Unifrac distances.*

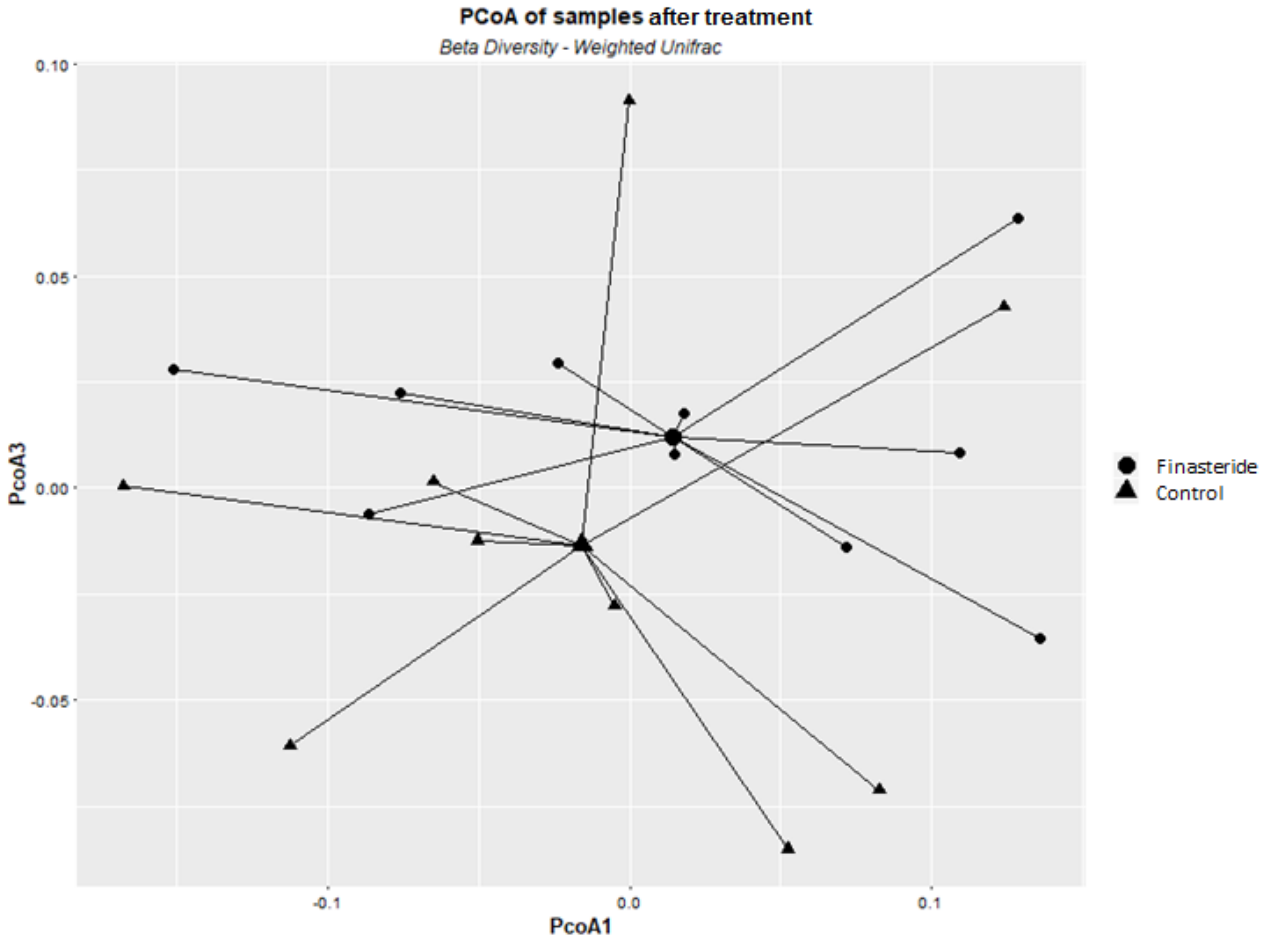

Figure 17*. Gut microbiota modulation at the end of finasteride treatment. Principal Coordinate Analysis (PCoA, beta-diversity) plot according to weighted Unifrac distances.*

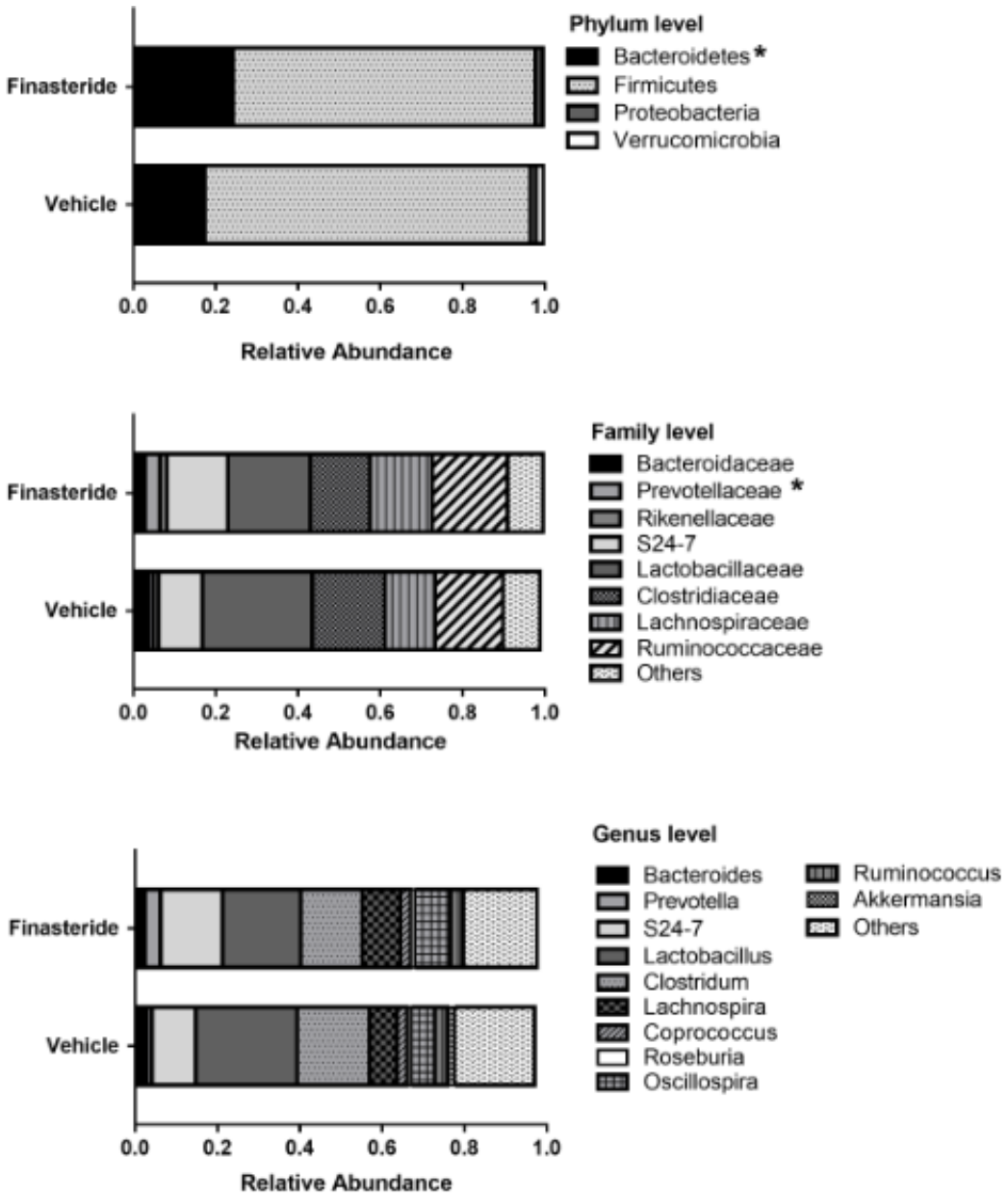

Figure 18. *Gut microbiota modulation at the end of finasteride treatment. Relative abundances in gut microbiota. Histograms of relative abundances at phylum, family, and genus. Statistical significant values (∗ $p < 0.05$) are reported highlighting the group with greater abundance.*

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) (Figure 19-20). 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 (Figure 21). 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) (Figure 21). 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 (Figure 21). *Oscillospira* genus, belonging to *Ruminococcaceae* family, and *Lachnospira* were significantly depleted in finasteride group (p=0.04, p=0.04 and p=0.03, respectively (Figure 21).

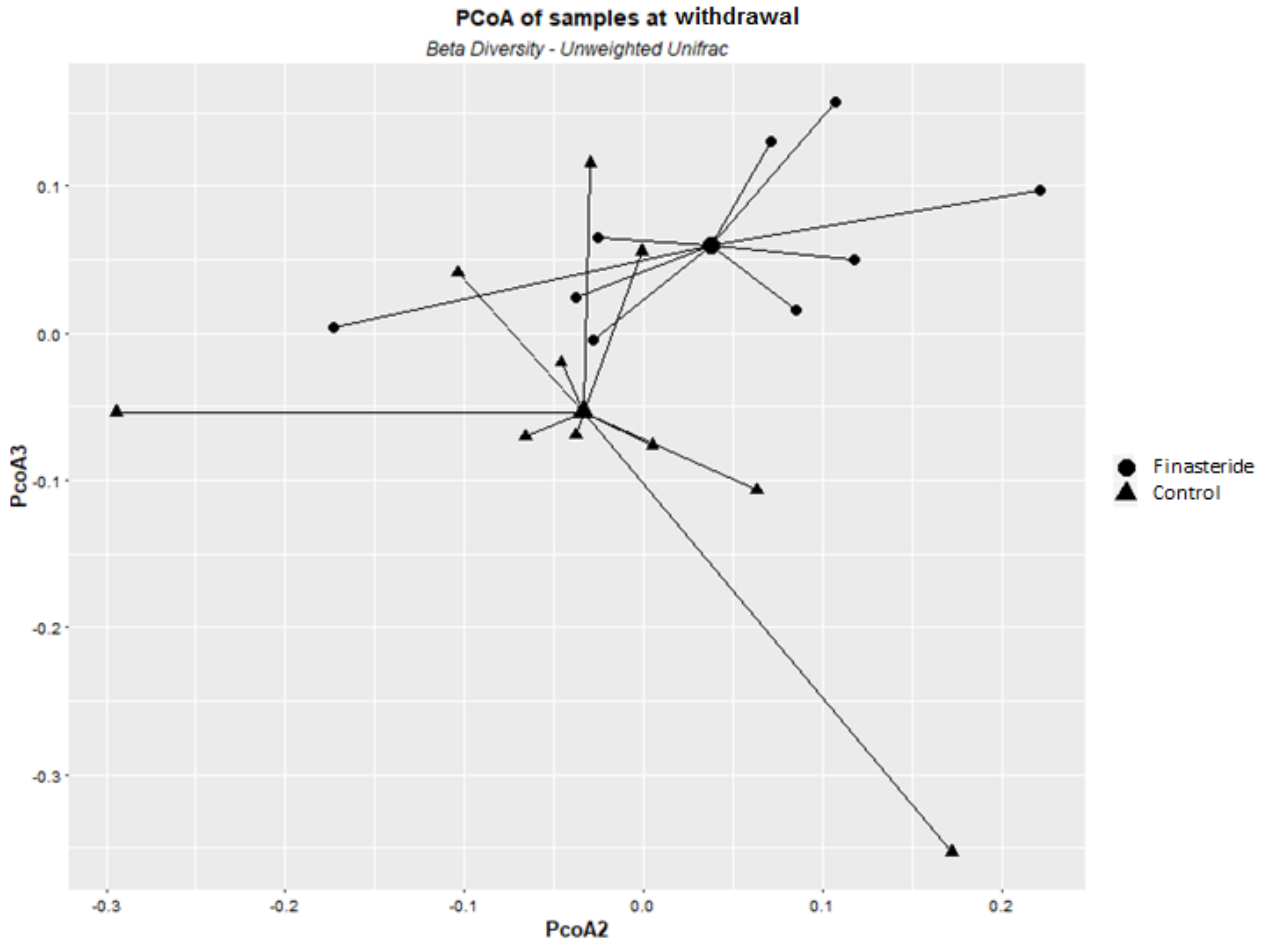

Figure 19. *Gut microbiota composition 1 month after finasteride withdrawal. Principal Coordinate Analysis (PCoA, beta-diversity) plot according to unweighted Unifrac distances.*

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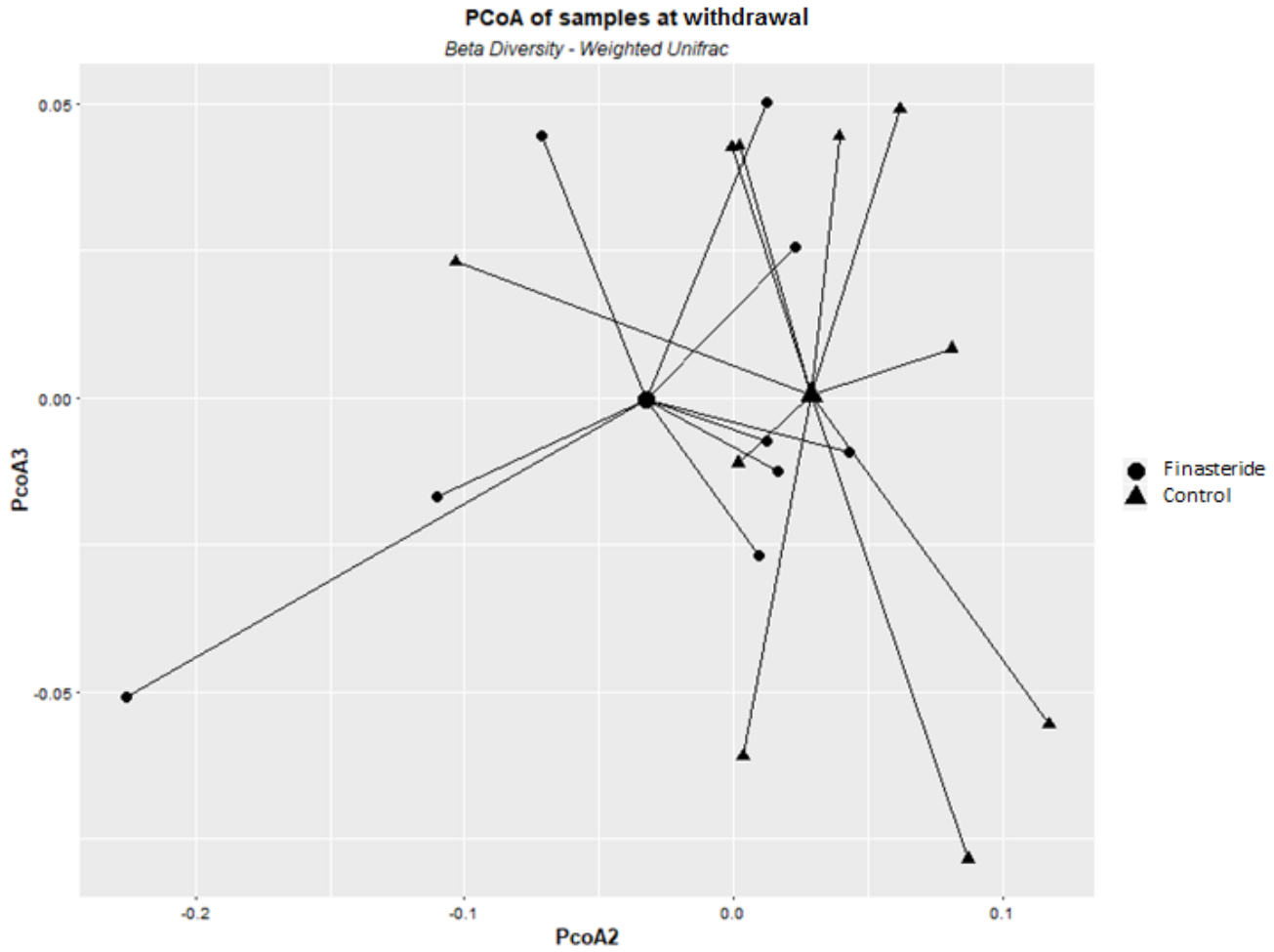

Figure 20. *Gut microbiota composition 1 month after finasteride withdrawal. Principal Coordinate Analysis (PCoA, beta-diversity) plot according to weighted Unifrac distances.*

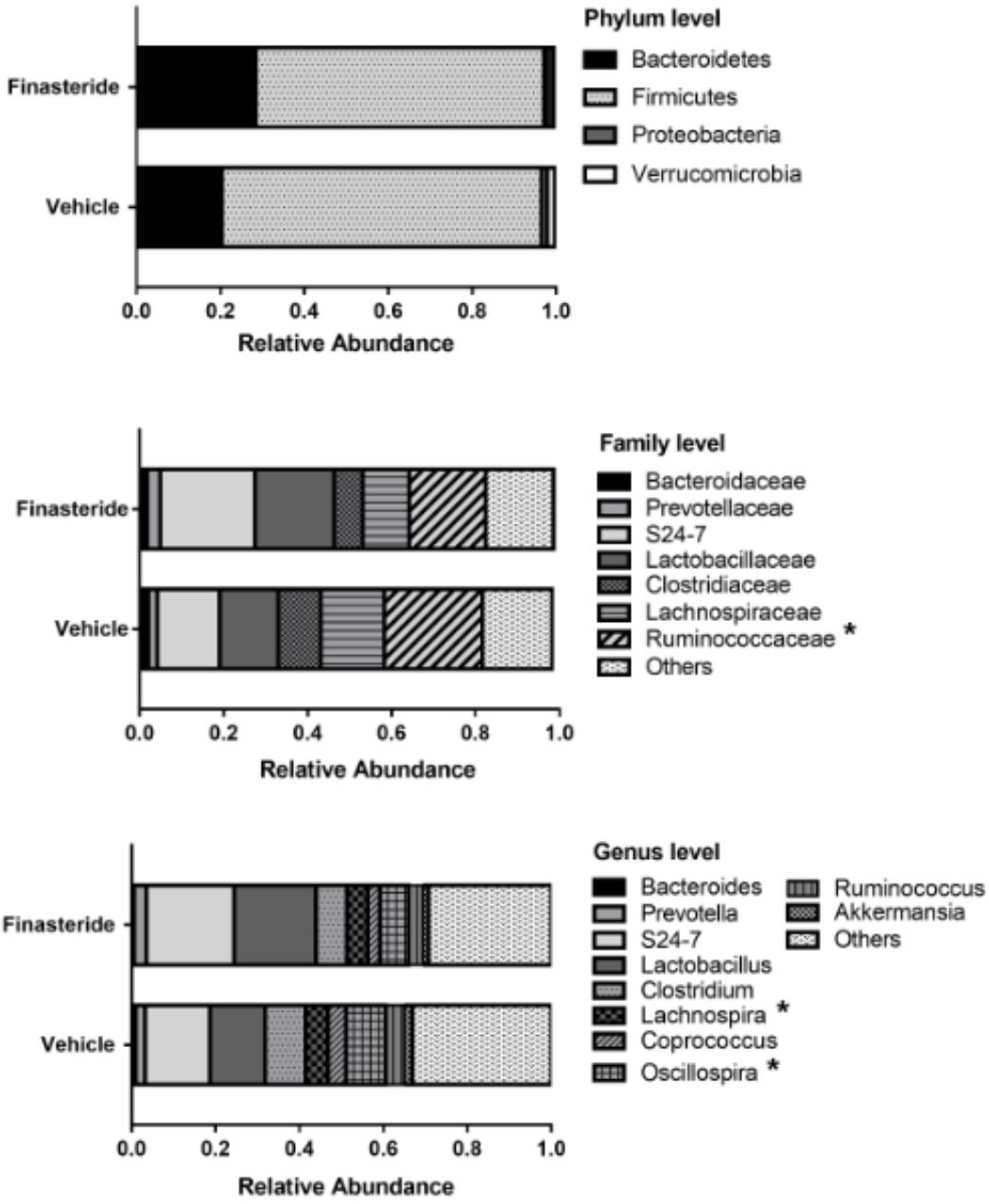

Figure 21. *Gut microbiota composition 1 month after finasteride withdrawal. Relative abundances in gut microbiota. Histograms of relative abundances at phylum, family, and genus. Statistical significant values (∗ $p < 0.05$) are reported highlighting the group with greater abundance.*

5. DISCUSSION

Data here obtained show 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 it 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). This debate is interesting but beyond the scope of the present discussion, 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 major depressive disorder (MDD), impairments in emotional processing lead to biases in cognitive processes (Murphy *et al*., 1999, Robinson *et al*., 2011, Roiser *et al*., 2012). The importance of these biases in MDD was first proposed by Beck in his cognitive theory of depression (Beck, 1976). The accumulation of studies of the psychological and biological aspects of depression has reached a critical mass warranting a new synthesis. The finding of relationships among the different genetic, environmental, neurophysiological and cognitive aspects of the vulnerability to and development of depression call for future studies integrating these observations into a more comprehensible formulation. A series of multiple wave prospective studies could identify the relevant variables and their interrelationships. The studies ideally would be complementary to each other so that each finding would contribute to the overall formulation of the theory of depression (Beck *et al*., 2008). More recently, affective biases (i.e. intended as a tendency to process information so as to favor certain types of emotional valence) have been linked to the development, maintenance and treatment of MDD (Teasdale, 1983, Teasdale, 1988, Beck, 2008, Harmer *et al*., 2009a, Kircanski *et al*., 2012). Processes including attention, memory, emotional interpretation and decision making have been shown to be negatively biased in people suffering from MDD (Mathews and MacLeod, 2005; Clark *et al*., 2009; Gotlib and Joormann, 2010).

In our study, finasteride treatment increased immobility time in the forced swim test compared to vehicle treated animals. It is interesting to highlight that the effect of finasteride was not detected at

the end of the treatment, but at the withdrawal. This finding is in agreement with the persistent depressive symptomatology reported in a subset of PFS young male patients that received finasteride for the treatment of AGA (Giatti *et al*, 2018a; Melcangi *et al*, 2017).

In agreement with our potentially important results, in order to reinforce the behavioral aspects, it will be interesting in future studies, to investigate the psychological features including attention, memory, emotional interpretation and decision making.

Changes in hippocampal neurogenesis in rodents have been related with modifications in depressive-like behaviors (Santarelli *et al*, 2003). For instance, as reported by Snyder and coworkers (Snyder *et al*, 2011) decreased neurogenesis in the dentate gyrus is linked to increased immobility in the forced swim test. We here reported that 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 consistent 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 that we reported in the same 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 at the 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 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 agreement 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). It is interesting to note that, depressed patients, show alteration in hippocampal morphology, like for instance reduced volume, reduced dendritic complexity and neuronal soma size, as well as a 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). Indeed, microglia and astrocytes are the main mediators of the inflammatory response. In addition, 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 *et al*, 2012). Furthermore, reactive microglia are also less efficient in the maintenance of synaptic function (Tay *et al*, 2017). As here reported, 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 finding is consistent with an effect of finasteride on astrogliosis. In this context it is important to recall that, one month after finasteride withdrawal, a significant increase in the levels of DHP in the male rat hippocampus compared to control animals was reported (Giatti *et al*, 2016b). Interestingly, DHP is known to increase GFAP gene expression in astrocytes (Giatti *et al*, 2012b). Therefore, the increase in the number of GFAP immunoreactive astrocytes one month after finasteride treatment may be ascribed to an increase of DHP levels. 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.

On the other hand, finasteride treatment did not significantly modify 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 of astrocytes. Although no significant differences were detected in total number of microglia in the dentate gyrus among the different experimental groups, at the end of the treatment the number of microglial cells with a reactive phenotype was increased in the finasteride group compared to the vehicle group. This effect was not permanent, because one month after the end of the treatment most microglial cells showed a resting phenotype in both the finasteride and control groups. Thus, these observations suggest that, at least some features of microglial cells, might be influenced by the finasteride treatment. In this context, the significance of these morphological changes in microglia is unknown, but they may reflect a transition of resting microglia towards a pre-activated phenotype (type III). In addition, in finasteride-treated animals there was a considerable proportion of Iba1-immunoreactive cells with large somas and retracted and thicker processes (type IV); morphology that is characteristic of activated microglia (Diz-Chaves *et al*, 2012).

Reactive gliosis is associated with increased levels of pro-inflammatory molecules in the brain (Wang *et al*, 2018). In agreement with this finding, we here observed that 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 highlight that, analyzing these data by Student's t-test, a significant increase of the gene expression of this cytokine was reported at the withdrawal. In this context it is important to recall that the expression of both cytokines is increased in plasma of patients with depression and in the brain of animal models of depression (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 this thesis are still pertinent. Certainly, further investigation is needed to extend the effect of finasteride treatment and withdrawal described here to non-stressed subjects.

As here reported we observed that in agreement with the well know correlation of depression and plasma cytokines (Dowlati *et al*, 2010; Dunn *et al*, 2005; Maes *et al*, 1993a; Maes *et al*, 1993b; Maes *et al*, 1994) finasteride was able to affect, at least IL-1β levels, in our experimental model.

In particular, we observed that after subchronic treatment a decrease in the plasma levels of this cytokine was observed. A possible hypothesis for this anti-inflammatory effect of finasteride may be suggested by an increase of plasma estrogen levels. Indeed, as we previously demonstrated in our experimental model, the levels of 17β-E were significantly increased by finasteride in plasma, after drug treatment (Giatti *et al*, 2016b). In support to our hypothesis it is also important to highlight that inhibition of 5α-R by finasteride leads to the conversion of T to 17β-E, which produces salutary effects on the post-traumatic immune response (Frink *et al*, 2007). On the other hand, at the withdrawal we reported a decrease in the levels of this cytokine. Also this pro-inflammatory profile could be tentatively related to a decrease in neuroactive steroids. Indeed, as reported by Giatti and collaborators plasma levels of PROG, THP, DHT and 3alpha-diol were reduced at the finasteride withdrawal (Giatti *et al*, 2016b).

Data here reported also indicate for the first time that the subchronic treatment with this blocker of the enzyme 5α-R was able to affect gut microbiota of male rats. In particular, 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 finasteride in male rats are responsible for the gut microbiota modulation here observed (Giatti *et al*, 2016b).

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, as reported by Org and co-workers Ruminococcaceae are significantly affected by orchidectomy in mice (Org *et al*, 2016). On the other hand, as previously mentioned, using this experimental model, it has been observed that neuroactive steroid levels are not only altered in plasma, but also in different brain regions (Giatti *et al*, 2016b). The existence of a gut microbiota-brain axis has been recently proposed (Mayer, 2011; Sharon *et al*, 2016), therefore 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. An alternative possibility could be a direct action of finasteride on gut microbiota. Indeed, 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). In addition, it is also interesting to highlight that changes in gut microbiota we here observed are very similar to what was observed in patients with major depressive disorder (Jiang et al., 2015) as well as in animal models of depression. For instance, as reported by Yu and collaborators, 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*, 2016b) 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 two DHP metabolites, such as THP and isopregnanolone, that are able to modulate the activity of GABA-A receptor (Giatti *et al*, 2016b). This neurotransmitter 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 was able to counteract depression-related behavior by directly increasing the levels of this neurotransmitter and, indirectly through the vague nerve, by modulating its 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.

6. CONCLUSION

In conclusion, observations reported in this thesis indicate that finasteride treatment leads to several alterations in the hippocampus, both after treatment and at its withdrawal.

In particular, at the end of finasteride treatment, increased proliferation in the subgranular zone of the dentate gyrus, increased number of microglia with reactive phenotype in the hilus and increased mRNA levels of TNF-α were observed. Moreover, other changes by one month after finasteride withdrawal, such as decreased proliferation in the subgranular zone, decreased granule cell density in the granule cell layer, 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 were detected. Interestingly, 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.

In addition, peripheral inflammation assessment revealed, after drug treatment, a significant decrease in plasma levels of IL-1β in finasteride-treated vs control animals. On the other hand, in agreement with literature and with the depressive-like behavior, a significant increase in plasma levels of IL-1β in finasteride-treated vs control animals at withdrawal period was observed. These observations seem to support a role of peripheral inflammation in addition to what we reported on neuroinflammation.

Furthermore, it was also observed for the first time that the subchronic finasteride treatment was able to affect gut microbiota of male rats. In particular, after treatment, 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.

As previously reported, important changes in the levels of neuroactive steroids are detected in the hippocampus and other brain regions by finasteride treatment as well as by one month after its withdrawal (Giatti *et al*, 2016b). Therefore, since neuroactive steroids regulate neurogenesis, gliosis and neuroinflammation as well as inflammation (Galea, 2008; Giatti *et al*, 2012a; Melcangi *et al*, 2016) and since PFS patients also show changes in neuroactive steroid levels and depressive symptomatology (Caruso *et al*, 2015b; Melcangi *et al*, 2013b; Melcangi *et al*, 2017), we proposed that the effect of finasteride on depression, neuroactive steroid levels, neurogenesis, neuroinflammation/inflammation and gut-microbiota composition could be interrelated events.

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