Steroid 5α-reductase isozymes in the adult female rat brain: central role of dihydrotestosterone

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Abstract

The enzyme 5α-reductase (5α-R) (EC 1·3·99·5) exists as two isoforms, 5α-R type 1 (5α-R1) and 5α-R type 2 (5α-R2). 5α-R1 has been associated with catabolic functions whereas 5α-R2 has been associated with sexually dimorphic functions of the male. We recently demonstrated that both 5α-R isozymes are present in the central nervous system (CNS) of the adult male rat and are regulated in an opposing way by androgens. This finding raises the question as to whether both isozymes play a role in the sexual dimorphism of the CNS, besides other functions. To test this hypothesis, it is essential to study the regulation of both isozymes by androgens in the female. In this work, we studied the effects of testosterone (T) and dihydrotestosterone (DHT) on mRNA levels of both 5α-R isoforms in the prefrontal cortex of the adult female rat by one-step quantitative RT-PCR coupled with laser-induced fluorescence capillary electrophoresis. Our results demonstrate for the first time that 5α-R2 mRNA is slightly regulated by T and DHT in females. Surprisingly, 5α-R1 mRNA is not regulated by T in the intact female, whereas it is very positively regulated by DHT, a more potent androgen than T. These data indicate the great sexual dimorphism in the CNS with respect to both 5α-R isozymes, and suggest a crucial role of DHT in the sexual dimorphism of the CNS in the female. These results open up a new research line that may lead to a better understanding of the physiology of the CNS.

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Introduction

The physiological importance of 5α-reductase (5α-R) (EC 1·3·99·5) in the brain may derive from two of its properties: its capability to convert testosterone (T) to a more potent androgen, dihydrotestosterone (DHT), that appears to participate in the sexual differentiation processes of some brain regions (Lauber & Lichtensteiger 1996, Poletti *et al.* 1997,1998*b*, Melcangi *et al.* 1998, Torres & Ortega 2003*a*); and its capability to convert progesterone and deoxycorticosterone (DOC) to their respective 5α-reduced derivatives, precursors of allopregnanolone and tetrahydroDOC, potent allosteric modulators of the γ-aminobutyric acid receptor ($GABA_A$-R) (Mellon & Griffin 2002), which participates in the regulation of various psychophysiological phenomena (Purdy *et al.* 1991, Majewska 1992, Melcangi *et al.* 2005, Patte-Mensah *et al.* 2005). The enzyme 5α-Reductase (5α-R) (EC 1·3·99·5) exists as two isoforms, 5α-R type 1 (5α-R1) and 5α-R type 2 (5α-R2) and both are present in the brain. The predominant mRNA species in rat brain is 5α-R1 (Lephart 1993), which has been proposed as a constitutive enzyme that essentially plays a catabolic and neuron protective role (Poletti *et al.* 1998*b*). 5α-R2 participates in sexually dimorphic functions of the male, such as in the development of prostate and external genitalia and in the differentiation of the CNS (Russell & Wilson 1994, Poletti *et al.* 1998*b*).

In order to determine the brain regions as well as the nerve cell types which contain 5α-R, Pelletier *et al.* (1994) studied the immunocytochemical localization of the enzyme, finding the immunoreactive material located in several brain areas including hypothalamus, amigdala, hippocampus, cerebellum and cerebral cortex. Moreover, Melcangi *et al.* (1993) reported 5α-R activity in primary cell cultures of neurons, oligodendrocytes, and astrocytes obtained from rat brain.

We recently demonstrated that these 5α-R isozymes are regulated in the male CNS in an opposing manner by androgens (Torres & Ortega 2003*a*), suggesting that both isozymes may play a role in the sexual dimorphism of the CNS, besides other functions (Paul & Purdy 1992, Torres & Ortega 2003*b*). We proposed that 5α-R2 participates in masculinization processes in male rats, whereas 5α-R1 may be involved in the feminization of the brain. In order to test this hypothesis, it is of maximal interest to know the mRNA levels of both 5α-R isozymes in the brain of female rats and their regulation by androgens, determining sexually dimorphic differences in these isozymes. To our knowledge, this issue has not been addressed in the literature.

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Table 1 Primer sequences and PCR products

	Primer sequence (5′–3′)	**Size** (bp)
Name		
R1-F	GAGATATTCAGCTGAGACCC	185
R1-R	TTAGTATGTGGGCAGCTTGG	
R2-F	ATTTGTGTGGCAGAGAGAGG	192
R2-R	TTGATTGACTGCCTGGATGG	
IS1-F	GAGATATTCAGCTGAGACCCACGTAAACGCCCACAAGTTC	300
IS1-R	TTAGTATGTGGGCAGCTTGGTCTTGTAGTTGCCGTCGTCC	
IS2-F	ATTTGTGTGGCAGAGAGAGGACGTAAACGGCCACAAGTTC	300
IS2-R	TTGATTGACTGCCTGGATGGTCTTGTAGTTGCCGTCGTCC	

Analysis of mRNA levels of specific genes may allow an estimation of gene expression. The present paper aimed to study 5α-R isozymes mRNA levels and their regulation by T and DHT in the prefrontal cortex of adult female rats, using a method that combines the high specificity of one-step quantitative RT-PCR with the sensitivity of laser-induced fluorescence capillary electrophoresis (LIF-CE). In this study we have used the same experimental procedure that was previously reported in males (Torres & Ortega 2003*a*) to enable a direct comparison of the results between males and females.

Materials and methods

Animals

Adult female Wistar rats weighing 180–200 g were housed in an air-conditioned room with fluorescent lights on from 7·00 to 1900 h, and were given standard laboratory pellet chow and water *ad libitum*. Experiments were made in strict accordance with the NIH guide for the Care and Use of Laboratory Animals. The experimental groups studied were: intact rats (I), intact rats plus T (I+T), intact rats plus DHT (I+DHT), ovariectomized rats (OVX), ovariectomized rats plus T (OVX+T) and ovariectomized rats plus DHT (OVX+DHT). Groups I+T and OVX+T were injected s.c. with oil vehicle (20% ethanol in sesame oil) containing T propionate (T_p; 1 mg/kg body weight/day) (George *et al.* 1991) on days 0, 3, 6, 9 and 12, a final injection was given 3 h before decapitation on day 15. To enable comparison of the effects of T and DHT, groups I+DHT and OVX+DHT were injected s.c. with oil vehicle (20% ethanol in sesame oil) containing DHT propionate (D_p; 1 mg/kg body weight/ day) (George *et al.* 1991) on the same days (days 0, 3, 6, 9, 12 and 15). I and OVX groups were injected s.c. on the same days with oil vehicle alone. The number of rats per group was 10. The animals were decapitated, and the brain was removed and weighed. Prefrontal cortex samples were frozen in liquid nitrogen and stored at −80 °C until analysis. Blood samples were collected in heparinized tubes. After coagulation, the blood was centrifuged at 800 ***g*** for 10 min. The plasma was separated and stored at −20 °C until the hormonal measurements were performed.

Hormone assays

Plasma T concentrations were measured by RIA using a commercial DiaSorin (Vercelli, Italy) kit without modification. The intra- and inter-assay coefficients of variation were 7·6% and 12·0%, respectively, the sensitivity was 0·05 ng/ml, and the cross-reactivity of the antiserum was 6·9% for DHT. Plasma DHT concentrations were measured by direct ELISA (Diagnostic Biochem Canada Inc., Ontario, Canada). The intra-and inter-assay coefficients of variation were 5·9% and 7·5%, respectively, the sensitivity was 6·0 pg/ml, and the cross-reactivity of the antiserum was 8·7% for T.

Oligonucleotides used for amplifications

Sequences of rat 5α-R isozymes were obtained from GeneBank and the sequence of plasmid pEGFP-C1 was obtained from the Clontech web page (www.clontech.com). These sequences were used to design the primer pairs. Primers for 5α-R isozymes were 20 bp of length, whereas primers used to synthesize both competitor molecules were 40 bp of length. All forward primers were end-labeled with 6-carboxy-fluorescein. Oligonucleotides were synthesized by PE-Applied Biosystems, UK. Primer sequences (5′-3′) and PCR product sizes are presented in Table 1.

Construction of the internal standard template

Two synthetic internal standard (IS) DNAs of 300-bp were synthesized from the sequence of plasmid pEGFP-C1 (Clontech, Palo Alto, CA, USA) following Torres and Ortega (2004*a*). Both competitive molecules, IS-1 (competitor DNA of 5α-R1) and IS-2 (competitor DNA of 5α-R2) were obtained after two consecutive amplifications from pEGFP-C1, with 5′ and 3′ ends

modified to contain the same nucleotide sequences as SRD5A1 or SRD5A2 (Torres & Ortega 2004*a*).

RT-PCR

Total RNA was extracted from 25 mg of rat prefrontal cortex tissues by acid-guanidinium thiocyanate-phenol-chloroform (Chomczynski & Sacchi 1987). The RNA was resuspended in diethyl pyrocarbonate-treated water and quantitated spectrophotometrically for analysis. First-strand cDNA was carried out according to Torres *et al.* (2004). The PCR profile was: denaturing, 94 °C for 30 s; annealing, 55 °C for 30 s; and extension, 72 °C for 30 s. In each case the number of cycles was 35. PCR was carried out in a Perkin-Elmer 2400 Thermal Cycler.

Analysis of PCR products

A CE system with LIF detection was used to characterize RT-PCR products. After amplification, an aliquot of the sample (1 μl) was diluted 1/20 with 18·5 μl of formamide and 0·5 μl of GeneScan-500 TAMRA Size Standard (Applied Biosystem, Warrington, UK) and denatured at 95 °C for 3 min. Capillary electrophoresis was carried out in a 47 cm-silica capillary containing POP-4 polymer (Applied Biosystem, Branchburgh, NJ, USA). The separation capillary was first filled with the polymer solution. The sample was then injected into the separation capillary for 5 s. The temperature of the separation capillary was 60 °C and each sample ran for 24 min at 100 V/cm. We performed LIF-CE in an ABIPRISM 310 Genetic Analyzer (Applied Biosystem).

The ratios of fluorescence of both 5α-R/IS were plotted against the amount of the appropriate competitive DNA, and the concentration of target DNA in the sample was calculated according to Torres & Ortega (2004*a*). The concentration of problem cDNA was corrected by the correction factor K. The correction factor K depends on the RT-PCR characteristics and is the product of three components that represent the correction due to the difference in size between problem and standard; the correction due to the addition of the internal standard in DNA form, and the efficiency of retrotranscription (Torres & Ortega 2004*a*).

Statistical analysis

Statistically significant differences between the groups were analyzed by a two-way ANOVA. The Bonferroni method was used in this study. The SPSS version 9·0 for Windows software package was used in the statistical analysis. Results are expressed as mean ± S.E.M.

Results

Serum hormonal levels

We found that the T levels in OVX animals (0·2 ± 0·09 ng/ml) were lower than those in I animals (0·3 ± 0·08 ng/ml). After T treatment, there was a significant increase in T levels in both I (10·26 ± 0·5 ng/ml, $P<0·001$) and OVX (9·5 ± 0·5 ng/ml, $P<0·001$) rats in comparison with their pre-treatment levels.

We found that the DHT levels in OVX animals (49 ± 11 pg/ml) were lower than those in I animals (78 ± 21 pg/ml). After DHT treatment, there was a significant increase in DHT levels in both I (750 ± 60 pg/ml, $P<0·001$) and OVX (500 ± 40 pg/ml, $P<0·001$) animals in comparison with their respective pre-treatment levels.

Quantification of 5α-R1 mRNA levels in prefrontal cortex

The amount of mRNA was expressed as number of mRNA copies per 100 ng of total RNA. After cDNA was generated from total RNA by RT reaction, it was co-amplified in the presence of decreasing amounts of the competitive DNA (64×10^6–$0·5 \times 10^6$ molecules). We co-amplified 5α-R1 cDNA and the competitive standard DNA IS-1 using the same pair of primers. With decreasing amounts of the competitive DNA, the relative intensity of amplified product of target DNA increased.

The mean amount of 5α-R1 mRNA in the prefrontal cortex of the different experimental groups is displayed in Fig.1. The 5α-R1 mRNA levels in OVX animals were 1·4-fold than in I animals. After T treatment, there was not a significant increase of 1·04-fold in I rats in comparison with its respective pre-treatment levels. After DHT treatment, there was a significant increase of 4·5-fold in I rats in comparison with its respective pre-treatment levels. After T and DHT treatment, there was a significant increase in 5α-R1 mRNA levels in OVX animals in comparison with their respective pre-treatment levels (1·7-fold for T and 2·9-fold for DHT, respectively).

Quantification of 5α-R2 mRNA levels in prefrontal cortex

In the same way, we co-amplified 5α-R2 cDNA and the competitive standard DNA IS-2 using the same pair of primers. With decreasing amounts of the competitive DNA, the relative intensity of amplified product of target DNA increased. Thus, the ratio of fluorescence of 5α-R2/IS-2 was plotted against the amount of competitive DNA IS-2.

The mean amount of 5α-R2 mRNA in the prefrontal cortex of the different experimental groups is shown in

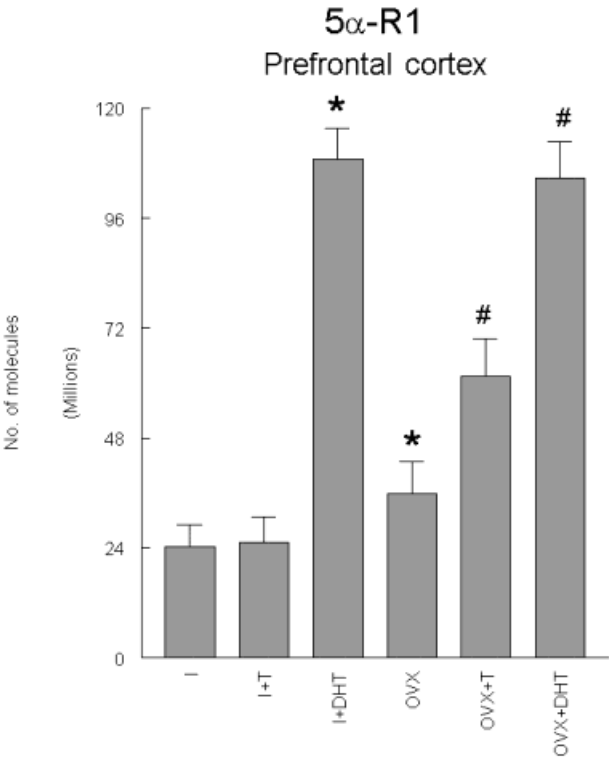

Figure 1 Effects of testosterone (T) and dihydrotestosterone (DHT) on steroid 5α-reductase type 1 (5α-R1) mRNA levels of intact (I) and ovariectomized (OVX) animals in prefrontal cortex of adult female rat. *$P<0{\cdot}01$ or less versus I animals. #$P<0{\cdot}01$ or less versus OVX animals.

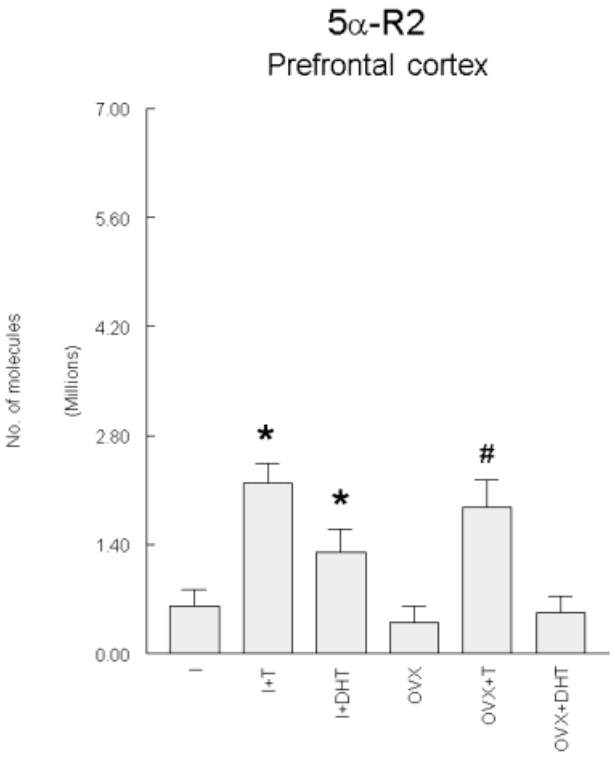

Figure 2 Effects of testosterone (T) and dihydrotestosterone (DHT) on steroid 5α-reductase type 2 (5α-R2) mRNA levels of intact (I) and OVX animals in prefrontal cortex of adult female rat. *$P<0{\cdot}01$ or less versus I animals. #$P<0{\cdot}01$ or less versus OVX animals.

Fig. 2. The 5α-R2 mRNA levels in I animals were 1·5-fold than in OVX animals. After T treatment, there was a significant increase in 5α-R2 mRNA levels in both I and OVX animals, in comparison with their pre-treatment levels (3·5-fold for I and 4·6-fold for OVX animals, respectively). After DHT treatment, there was an increase in 5α-R2 mRNA levels in both I and OVX animals in comparison with their respective pre-treatment levels. This increase was only significant in I animals (2·0-fold for I and 1·2-fold for OVX animals, respectively).

Discussion

Determination of the mRNA levels of specific genes may allow an estimation of gene expression. The present paper aimed to study the mRNA levels of both 5α-R isozymes and their regulation by T and DHT in the prefrontal cortex of adult female rats. The results of our experiments demonstrated that both 5α-R mRNAs are expressed in prefrontal cortex of the adult female rat and that 5α-R1 is much more abundant than 5α-R2 (Lephart 1993). This wide disparity (abundance of 5α-R1 mRNA was 40-fold that of 5α-R2) may have physiological relevance. Although both isozymes have affinity for the same substrates (progesterone>testosterone>androstenedione>corticosterone), the affinity of 5α-R1 is much lower than that of 5α-R2 (Negri-Cesi *et al.* 1996). According to their respective V_{max} values, 5α-R1 has a much greater capacity to reduce these substrates compared with 5α-R2 (Negri-Cesi *et al.* 1996). The values of these kinetic parameters indicated that 5α-R1 could only act when steroid levels were high. The high V_{max} of 5α-R1 endows it with a great capacity to reduce steroids at 5α, and for this reason 5α-R1 has been associated with purely catabolic actions, protecting neurons from excess of glucocorticoids that may induce apoptotic processes (Mahendroo *et al.* 1997, Poletti *et al.* 1998*a*). In contrast, a substantially high 5α-R2 mRNA levels was found in spinal cord (SC) (Pozzi *et al.* 2003), an observation perfectly in agreement with recent findings that indicate 5α-R2 immunoreactive material in adult rat SC is much higher than those of 5α-R1 (Patte-Mensah *et al.* 2004).

Despite the reported association of 5α-R2 with brain masculinization processes (Poletti *et al.* 1998*b*), our results demonstrate that both 5α-R mRNAs are present in the

prefrontal cortex of adult female rats with a higher abundance (3·6-fold for 5α-R1 and 2·2-fold for 5α-R2) than those previously observed by our group in adult males (Torres & Ortega 2003*a*). However, no sex difference was observed in the distribution of 5α-R1 and 5α-R2 immunoreactive elements in the SC of adult female and male rats (Patte-Mensah *et al.* 2004).

According to the previous findings observed in the SC (Patte-Mensah *et al.* 2004) the 5α-R2 mRNA levels were not significantly modified two weeks after castration in the prefrontal cortex of female rats. 5α-R2 mRNA is much less regulated by T (5-fold and 26-fold in I and castrated animals, respectively) and DHT (3-fold and 13-fold in I and castrated animals, respectively) in females than in males (Torres & Ortega 2003*a*). A possible explanation may be that the steroid milieu during the neonatal period irreversibly imprints or programs 5α-R2 expression, as this occurs with other hepatic enzymes (Gustafsson & Stenberg 1974*a,b*). Another possible explanation may be genetic differences in 5α-R2 between males and females. Our findings are in accordance with previous reports that treatment with an androgen receptor (AR) blocker, flutamide, produces a significant decrease in the 5α-R2 mRNA levels in the brain of male animals, whereas it is less effective in modulating the expression of this isoform in female brain (Poletti *et al.* 1998*b*). In our opinion, the slight regulation of 5α-R2 by androgen suggests that the production of a large amount of DHT may not be the main function of this isozyme in the female, unlike in the male.

We consider our 5α-R1 results to be of major interest. The mRNA levels of this isozyme are highly regulated by DHT in both I and OVX animals, and are not regulated by T in I females. These results are surprising, because it is known that both T and DHT bind to AR, although the affinity of DHT is four-fold that of T.

There are various possible explanations of our findings, including: a) The long-established aromatization of T to estradiol in the brain (MacLusky & Naftolin 1981). Thus, T and DHT may exert different physiological effects, given that DHT would act via AR and T would act via estrogen receptors. Unfortunately, the hormonal levels in the cerebral cortex have not been measured in this work. It is generally accepted that the aromatase enzyme depends on the androgenic status of animals. Thus, castration decreases aromatase activity and mRNA levels, whereas T treatment restores them (Harada *et al.* 1992, Abdelgadir *et al.* 1994). Cytochrome P450 aromatase has been found in neurons from the cerebral cortex of neonatal rats (Zwain & Yen 1999). However, other authors have been found that aromatase is mainly localized in specific brain areas such as the hypothalamus–preoptic area but not in the cerebral cortex (Lephart 1996, Kato *et al.* 1997). *In vitro* studies have also demonstrated that T increases hypothalamic but not cortical aromatase (Beyer *et al.* 1994). Given that we administered T and DHT systemically, the peripheral aromatization of T should be borne in mind. Nevertheless, the females show higher hepatic 5α-R activity and mRNA levels compared with males (Gustafsson & Stenberg 1974*a,b*, Torres & Ortega 2003*c*, 2004*b*); thus, the aromatization of T would be greater in males than in females. Hence, the differential effects of T and DHT on 5α-R1 found in the present study cannot be attributed solely to the conversion of T to estradiol but rather indicate a sexually dimorphic regulation of the enzyme. b) The possible metabolism of DHT into inactive 3β-androstanediol (3β-Diol) or active 3α-androstanediol (3α-Diol), as occurs in the pituitary (Denef *et al.* 1974). In general, reduction at the C3 position decreases the binding affinity to intracellular receptors (Negri-Cesi *et al.* 1996) such as AR. The enzyme 3α-hydroxyesteroid oxidoreductase (3α-HSOR) is present in the cerebral cortex, although the formation of 3α-Diol is generally lower in the cerebral cortex than in white matter (Negri-Cesi *et al.* 1996). c) Finally, the differential effect of T and DHT on 5α-R1 may be due to the existence of different classes of androgen-responsive elements (Russell & Wilson 1994) and different signaling pathways. The differences observed in the response to androgens between I and OVX rats could be attributed to the influence of some ovarian factors. Perhaps, the lack of ovaries may compensate for a different hormonal background, allowing for direct downregulation of T- or DHT-modulated transcription factors such as AR.

5α-R1 mRNA in the female is drastically increased by DHT in an opposing way to that observed previously by our group in the male (Torres & Ortega 2003*a*). These data demonstrate that 5α-R1 presents a major sexual dimorphism in the brain, at least in the prefrontal cortex, and may therefore be involved in sexual dimorphism throughout the female's life. 5α-R1 has to date been considered a constitutive enzyme associated with purely catabolic actions. However, the present results broaden this concept, because 5α-R1 mRNA was shown to be highly and positively regulated by DHT, an steroid lacking $\Delta^{4,5}$ double bond. Furthermore, the mRNA profile of 5α-R1 in the cerebral cortex of the adult female rat is different from its profile in the liver (Torres & Ortega 2004*b*), which is the catabolic organ *par excellence*.

If the function of 5α-R1 in the female is not solely catabolic and the function of 5α-R2 in the female is not the production of the potent androgenic hormone DHT, the question arises as to the function of these isozymes in the female brain. One possibility is that, in addition to the catabolic effects of 5α-R1, both 5α-R isozymes intervene in the production of a 5α-reduced progesterone derivative, this hypothesis is supported by several findings. 5α-R2 mRNA is slightly regulated by

androgens, as we report in this paper. 5α-R2 mRNA has been induced in the hippocampus of female mice by progesterone, producing 5α-reduced metabolites (Matsui *et al.* 2002). Both 5α-R isozymes have a higher affinity for progesterone than for other steroid substrates. Circulating progesterone levels are higher in the female than in the male.

The 5α-reduced steroid may be transformed by action of the enzyme 3α-HSOR, also present in the CNS, into the 3α5α-reduced progesterone derivative allopregnanolone. This compound is a potent neurosteroid whose action is mediated by allosteric modulation of the $GABA_A$-R complex (Mellon & Griffin 2002). Neurosteroids are steroids produced within the nervous system of vertebrates (Baulieu 1998, Mensah-Nyagan *et al.* 1999, Patte-Mensah *et al.* 2003) which are involved in the regulation of stress responses, anxiety, sleep, aggressive behavior and other important neurobiological processes in the CNS and peripheral nervous system (Purdy *et al.* 1991, Majewska 1992, Melcangi *et al.* 2005, Patte-Mensah *et al.* 2005).

We previously reported that the neonatal administration of the GABA agonist diazepam to male rats feminizes behavior and CNS structures (Segovia *et al.* 1996,1999). 3α5α-reduced neurosteroids regulate $GABA_A$ receptors in a similar way to barbiturates (Majewska 1992, Paul & Purdy 1992) and may therefore exert similar effects in the CNS, favoring the formation and maintenance of female brain structures. This would offer a biological explanation for the greater levels of 3α5α-reduced neurosteroids (Torres & Ortega 2003*b*) and of both 5α-R isozymes in the female versus male brain.

Our group previously demonstrated that DHT regulates 5α-R2 in the prostate and brain of male rats by a feed-forward mechanism (Torres & Ortega 2003*a*, Torres *et al.* 2003). We argued that 5α-R2 may act in the male as morphogen (George *et al.* 1991, Russell & Wilson 1994) throughout the life of the individual, favoring the maintenance of essentially masculine structures, this is consistent with the idea of DHT as androgenic. Now, we demonstrated for the first time that DHT positively regulates the mRNA levels of both 5α-R isozymes in the I female. Therefore, DHT could feminize brain structures (Valencia *et al.* 1992) by an increase in 3α5α-reduced neurosteroids through the induction of 5α-R isozymes.

To our best knowledge, our data provide the first evidence that both 5α-R isozymes are present in the brain of adult female rats, at least in the prefrontal cortex, and that their mRNA levels are regulated by androgens in a different way than in the male. Whereas 5α-R2 mRNA is slightly regulated by T and DHT, 5α-R1 mRNA is very positively regulated by DHT. In our opinion, both 5α-R isozymes may participate in the production of 3α5α-reduced neurosteroids, although 5α-R1 may act when the steroid levels are higher. The data showed in this work indicate the great sexual dimorphism in the CNS with respect to the two 5α-R isozymes and could point to their possible participation in the development and maintenance of sexually dimorphic structures throughout the life of the female. Interestingly, DHT, an essentially androgenic hormone, may feminize the CNS through 5α-R isozymes.

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