Effects of testosterone on brain mRNA levels of steroid 5α-reductase isozymes in early postnatal life of rat

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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), and both are present in the brain. 5α-R1 has been proposed as a constitutive enzyme that essentially plays a catabolic and neuron protective role whereas 5α-R2 has been associated with sexually dimorphic functions of the male. In this work, we studied the effects of testosterone (T), the masculinizing hormone of the central nervous system (CNS), on mRNA levels of both 5α-R isoforms in the prefrontal cortex of male and female rats during the postnatal sexual differentiation of the CNS in the rat, using one-step quantitative RT-PCR coupled with laser-induced fluorescence capillary electrophoresis (LIF-CE). We found an increase in 5α-R2 mRNA levels in both male and female rats after T treatment, while 5α-R1 mRNA levels were decreased in the same experimental conditions. Our results clearly indicated that T regulates the expression of both 5α-R1 and 5α-R2 genes in an opposite manner and independently of the sex. This could point to a crucial role of T in the sexual dimorphism for both 5α-R isozymes in the neonatal brain. These results open up a new research line that could improve understanding of the role of 5α-R isozymes in the physiology of the CNS.

Keywords: 5α-Reductase isozymes; Transcriptional regulation; Testosterone; Prefrontal cortex; Early postnatal life; Rat

1. Introduction

Previous studies by our group demonstrated expression of the two 5α-reductase (5α-R) isozymes in the brain of the adult male rat (Torres and Ortega, 2003), and it was hypothesized that both isozymes may play a decisive role in the sexual dimorphism of the CNS. Under physiologic conditions, the 5α-R2 isozyme might have an essentially masculinizing role in the brain of the male rat (Poletti et al., 1998a; Ribeiro and Pereira, 2005), as in the prostate (George, 1997; Russell and Wilson, 1994; Torres et al., 2003), converting testosterone (T) into the more potent androgen dihydrotestosterone (DHT) (Lauber and Lichtensteiger, 1996; Negri-Cesi et al., 1996; Poletti et al., 1997, 1998b). Although CNS masculinization in male rodents is known to be exerted by T after its ss aromatization to estradiol (Lephart, 1996; Negri-Cesi et al., 1996; Amateau and McCarthy, 2004), some brain structures specifically require the presence of DHT rather than T for their masculinization (Poletti et al., 1998b). In contrast, the 5α-R1 isozyme may play a demasculinizing/feminizing role in the CNS, producing 5α-reduced metabolites of progesterone that in turn generate allopregnanolone, (3α,5α-reduced metabolite of progesterone) by the action of 3α-hydroxysteroid oxidoreductase (3α-HSOR) (Paul and Purdy, 1992). Allopregnanolone is a potent neurosteroid that, similar to diazepam (DZ), modifies γ-aminobutyric acid type A receptor (GABA A-R) and may therefore exert a demasculinizing/feminizing effect in the CNS (Segovia et al., 1996).

If 5α-reductase isozymes are involved in the sexual dimorphism of the CNS, their regulation by androgens differ between male and female, as previously demonstrated by our group in adult animals (Torres and Ortega, 2003, 2006). Our group also reported the regulation profile of 5α-R isozymes by DHT in the prefrontal cortex during sexual differentiation of the CNS (Sánchez et al., 2005).

The question arises, given the above data, as to how the regulation profile of both 5α-R isozymes is exerted by T (masculine sexual hormone par excellence). The present study was designed to address this issue, investigating T regulation of 5α-R isozymes at the transcriptional level in the prefrontal cortex...
of male and female rats during the postnatal period of CNS sexual differentiation.

2. Experimental procedures

2.1. Animals

Male and female Wistar rats were used in this study. Rat pups and their mothers were housed in an air-conditioned room with fluorescent lights on from 7 a.m. to 7 p.m. A previously used protocol was followed (Sánchez et al., 2005) in order to compare the similarities or discrepancies between the effects of T and DHT (Sánchez et al., 2005) on 5α-R isozymes. Male pups were randomly divided into the following groups: (a) 10 males receiving a daily subcutaneous (s.c.) injection of oil vehicle (sesame oil) containing T (M + T); (b) 10 males receiving a daily s.c. injection of oil vehicle (M + V); (c) 10 males without treatment, as controls (M). Groups M + T and M + V were injected from day 5 through day 12 of postnatal life (PS-P12). T doses were 12 mg/kg body weight/day on days 5, 6, 7, and 8, and 15 mg/kg body weight/day on days 9, 10, 11, and 12. The same groups were established in the female pups: (a) 10 females receiving a daily s.c. injection of oil vehicle (sesame oil) containing T (F + T); (b) 10 females receiving a daily s.c. injection of oil vehicle (F + V); (c) 10 females without treatment, as controls (F). Groups F + T and F + V were injected from day 5 through day 12 of postnatal life (PS-P12). To enable comparison of the effects of T in males and females, female pups also received 12 mg/kg body weight/day on days 5, 6, 7, and 8, and 15 mg/kg body weight/day on days 9, 10, 11, and 12. The last injection was performed 2 h before decapitation. Experiments were made in strict accordance with the NIH guide for the “Care and Use of Laboratory Animals”.

To avoid possible adverse effects, the animals were decapitated without anesthesia on day 12. Blood samples were collected in heparinized tubes. After coagulation, the blood was centrifuged at 2000 rpm for 10 min. The plasma was separated and stored at −80 °C until hormonal measurements were performed. The brain was removed, frozen in liquid nitrogen, and stored at −80 °C until analysis. The dissection of prefrontal cortex areas was assessed with reference to the Atlas of Paxinos and Watson (1986). One thick slice of brain tissue was cut, demarcated by two coronal planes corresponding to the anterior/posterior position of Plates 7 (bregma 3.70 mm) and 10 (bregma 2.2 mm). Right and left hemispheres were divided by a sagittal cut and dorsal anterior cingulate cortex (ACd), prelimbic (PL) and infralimbic (IL) areas were then removed, using the shape of the subcortical white matter as the primary landmark.

2.2. 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. These sequences were used to design the primer pairs. Primers for 5α sequence of plasmid pEGFP-C1 was obtained from the Clontech web page. The 3′- and 5′-ends of the primers were modified to contain the same nucleotide sequences as SRD5A1 or SRD5A2 (Torres and Ortega, 2004).

2.3. Construction of the internal standard templates

Two synthetic internal standard (IS) DNAs of 300 bp were synthesized from the sequence of plasmid pEGFP-C1 (Clontech, Palo Alto, CA) as previously described (Torres and Ortega, 2004). 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 and Ortega, 2004).

2.4. Reverse transcription reaction-polymerase chain reaction

Total RNA was extracted from 25 mg of rat prefrontal cortex tissues by acid-guanidinium thiocyanate-phenol-chloroform. RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated water and spectrophotometrically quantitated 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.

2.5. 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. LIF-CE was carried out in an ABIPRISM 310 Genetic Analyzer (Applied Biosystems, USA) according to Torres et al. (2004) and Torres and Ortega (2004). 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 and Ortega (2004). The concentration of problem cDNA was corrected by the correction factor K. The correction factor K depends on the RT-PCR characteristics (Torres and Ortega, 2004).

2.6. Hormone assay

Plasma T concentrations were measured using an electrochemiluminescence immunoassay. The intra- and inter-assay coefficients of variation were 2.1 and 4.3%, respectively, and the sensitivity was 0.02 ng/ml.

2.7. Statistical analysis

Statistically significant differences among the groups were analyzed by a two-way ANOVA. The Bonferroni method was used in this study. The SPSS version 13.0 for Windows software package was used in the statistical analysis. Results are expressed as mean ± S.E.M.

3. Results

3.1. Quantitation of 5α-R1 mRNA levels in prefrontal cortex

The amount of mRNA was expressed as number of mRNA copies per microgram 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 to 0.5 × 10^6 molecules). 5α-R1 cDNA and the competitive standard DNA IS-1 were co-amplified using the same set of primers. With decreasing amounts of the competitive DNA, the relative intensity of amplified product of target DNA increased.

Fig. 1 depicts the mean amount of 5α-R1 mRNA in the prefrontal cortex of the different experimental groups. 5α-R1
mRNA levels were similar between female and male animals. There were no significant changes in 5α-R1 mRNA levels as a result of vehicle treatment either in males or females, in comparison with their respective controls. After T treatment, there was a significant decrease in 5α-R1 mRNA levels in both males and females in comparison with their respective controls.

3.2. Quantitation of 5α-R2 mRNA levels in prefrontal cortex

Likewise, 5α-R2 cDNA and the competitive standard DNA IS-2 were co-amplified 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 Fig. 2. 5α-R2 mRNA levels were significantly higher in the females than in the males. There were no significant changes in 5α-R2 mRNA levels as a result of vehicle treatment either in males or females in comparison with their respective controls. After T treatment, there was a significant and large increase in 5α-R2 mRNA levels in both males and females in comparison with their respective controls.

3.3. Comparison between 5α-R1 and 5α-R2 mRNA levels

In order to offer a better comparison of our results and given the wide disparity in 5α-R1 and 5α-R2 mRNA levels between control and T-treated animals, Table 1 details the results depicted in Figs. 1 and 2.

3.4. Plasma hormonal levels

The T levels in female pups were significantly lower than those in male pups \( p < 0.001 \). After T treatment, there was a significant increase in T levels in both male and female pups compared with their respective intact and oil vehicle-administered groups (Fig. 3).

4. Discussion

Determination of the mRNA levels of specific genes may allow an estimation of gene expression. The present paper aimed to study mRNA levels of both 5α-R isozymes and their regulation by T in the prefrontal cortex of male and female rats during the postnatal period of CNS sexual differentiation.

The results of our experiments demonstrated a marked increase in mRNA levels of 5α-R2 isozyme in both male and female rats after T administration. This profile is similar to that found in the prostate (Torres et al., 2003) and brain of the male and female adult rat (Torres and Ortega, 2003, 2006) and corresponds to a gene that is positively regulated by T and may therefore control masculine functions.

Previous experiments by our group (Torres and Ortega, 2003, 2006) showed a much higher regulation of 5α-R2 gene transcription by T in adult male versus female rats, supporting the hypothesis that this regulation is genetically sex conditioned (higher in male). According to the present findings, the positive

Table 1

<table>
<thead>
<tr>
<th></th>
<th>M</th>
<th>M + V</th>
<th>M + T</th>
<th>F</th>
<th>F + V</th>
<th>F + T</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-R1</td>
<td>123.77 ± 10</td>
<td>139.30 ± 11.5</td>
<td>29.58 ± 4.5</td>
<td>123.84 ± 9.8</td>
<td>125.23 ± 9.9</td>
<td>51.05 ± 5.8</td>
</tr>
<tr>
<td>5α-R2</td>
<td>(7.8 ± 0.7) × 10^{-3}</td>
<td>(9.60 ± 1.4) × 10^{-3}</td>
<td>26.16 ± 4.6</td>
<td>0.45 ± 0.05</td>
<td>0.47 ± 0.06</td>
<td>30.50 ± 5.1</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M.; \( ^* p < 0.001 \) vs. M animals, \( ^\dagger p < 0.001 \) vs. F animals. M, control male; M + V, male injected with oil vehicle; M + T, male injected with oil vehicle containing T; F, control female; F + V, female injected with oil vehicle; F + T, female injected with oil vehicle containing T.
effect of T on 5α-R2 mRNA is much greater in both male and female neonates than in the adult animal (Torres and Ortega, 2003, 2006). Moreover, 5α-R2 gene might be regulated by T to the same extent or even more in females as in males. The higher transcriptional regulation of 5α-R2 gene by T in adult rats is not sex dependent, and may result from differences in physiological T levels in male versus female rats during CNS sexual differentiation (Negri-Cesi et al., 2004).

Present results show that mRNA 5α-R1 levels during sexual differentiation of the CNS are lower after T administration in both male and female rats. This finding, which may appear surprising, is logical given that T is the primary male sex hormone and induces masculinizing events in the brain. Therefore, this study confirms the hypothesis that, besides its neuron protection functions (Poletti and Martini, 1999), the 5α-R1 isozyme may participate in demasculinizing/feminizing functions in the brain. This may occur via the synthesis of 3α,5α-reduced steroids of progesterone, which have a similar action to that of DZ on GABA\(_\Lambda\)-R and may therefore exert a demasculinizing/feminizing effect in the CNS (Segovia et al., 2005).

To our best knowledge, the present data provide the first demonstration of the opposite behavior of 5α-R1 and 5α-R2 genes after T administration during the CNS sexual differentiation of rats. Our results clearly indicate that the post-T administration behavior of both 5α-R1 and 5α-R2 genes is independent of the sex of the animal and may point to a crucial role for T in the sexual dimorphism of both 5α-R isozymes in neonatal brain.

During the period of CNS sexual differentiation in rat, regulation of 5α-R1 and 5α-R2 gene transcription by T differs from their regulation by DHT (Sánchez et al., 2005). Thus, 5α-R2 mRNA levels in the neonate female rat markedly increase after T administration and decrease after DHT administration (Sánchez et al., 2005). In the male neonate, 5α-R1 mRNA levels increase after DHT administration (Sánchez et al., 2005) and decrease after T administration. Interestingly, T increases 5α-R2 during CNS sexual differentiation in females, thereby enhancing masculinizing functions according to our hypothesis. In contrast, DHT, hitherto considered a metabolite of T with identical and more powerful functions, increases 5α-R1 in males during this period, which would enhance demasculinizing/feminizing functions in the CNS according to our hypothesis.

Although T administration (Torres and Ortega, 2003) increases the serum levels of both T and DHT (the latter was unfortunately not measured in this paper), the present results are not in disagreement with our previous findings (Sánchez et al., 2005). It appears likely that there are three classes of androgen-responsive genes: those that respond to the androgen receptor coupled with T, those that require the androgen receptor coupled to DHT, and those that respond to either ligand-bound receptor (Russell and Wilson, 1994). Furthermore, T may induce the neuronal enzyme aromatase in the brain, and most of the effects of T in the sexual dimorphism of the CNS are known to be exerted via estrogen receptor after their local aromatization to estradiol (Lephart, 1996; Negri-Cesi et al., 1996; Amateau and McCarthy, 2004).

According to the present findings and previous reports by our group, T and DHT play a key role in the sexual dimorphism of the 5α-R in the brain during postnatal differentiation of the rat CNS. The opposite effects exerted by T and DHT on 5α-R isozymes in the neonatal brain give DHT a hormonal role that is independent of the role of T and may in some cases oppose it.

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