PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 69 (1999) 117–122

The Journal of Steroid Biochemistry & Molecular Biology

Androgen-activating enzymes in the central nervous system☆

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Abstract

In the rat brain, several steroids can be converted by specific enzymes to either more potent compounds or to derivatives showing new biological effects. One of the most studied enzyme is the 5α-reductase (5α-R), which acts on 3keto-Δ4 steroids. In males, testosterone is the main substrate and gives rise to the most potent natural androgen dihydrotestosterone. In females, progesterone is reduced to dihydroprogesterone, a precursor of allopregnanolone, a natural anxiolytic/anesthetic steroid. Other substrates are some gluco- and minero-corticoids. Two isoforms of the 5α-R, with limited degree of homology, have been cloned: 5α-R type 1 and type 2. The 5α-R type 1 possesses low affinity for the various substrates and is widely distributed in the body, with the highest levels in the liver; in the brain, this isoform is expressed throughout life and does not appear to be controlled by androgens. 5α-R type 1 in the rat brain is mainly concentrated in myelin membranes, where it might be involved in the catabolism of potentially neurotoxic steroids. The 5α-R type 2 shows high affinity for the various substrates, a peculiar pH optimum at acidic values and is localized in androgen-dependent structures. In the rat brain, the type 2 isoform is expressed at high levels only in the perinatal period and is controlled by androgens, at least in males. In adulthood, the type 2 gene appears to be specifically expressed in localised brain regions, like the hypothalamus and the hippocampus.

The 5α-R type 2 is present in the GT1 cells, a model of LHRH-secreting neurons. These cells also contain the androgen receptor, which is probably involved in the central negative feedback effect exerted by androgens on the hypothalamic–pituitary–gonadal axis. The physiological significance of these and additional data will be discussed.

Keywords: Testosterone; 5α-reductase; Flutamide; Neurons; LHRH; GT1 cells; Rat brain

1. Introduction

Several central nervous system (CNS) functions are regulated by gonadal steroids; a typical example of this is provided by the control of the endocrine and neuronal activities involved in the modulation of the prenatal sexual differentiation of the brain, of adult sexual behaviour, of gonadotropin secretion, etc.; these processes occur via the interaction of androgens and/or estrogens with the respective intracellular steroid receptors. Both types of receptors can be modulated by androgens, which may act directly or after having been transformed to estrogens by the action of the neuronal enzyme aromatase. The androgenic compound testosterone produced by the testis, is also the precursor of the most potent natural androgenic steroid dihydrotestosterone (DHT); the transformation of testosterone into DHT is achieved by the action of the enzyme 5α-reductase (5α-R) and plays clear physiological roles in the peripheral androgen-dependent structures; for instance it is involved in the control of development and function of the prostate and of the seminal vesicle; it is responsible for hair and beard growth, etc. The intracellular formation of DHT precedes the process of activation of the androgen receptor (AR), an essential step for the receptor-mediated transcription of androgen-responding genes; this provides a mechanism of amplification of the androgenic signal because of the higher affinity of DHT for the AR (four times that of testosterone) [1–4]. Moreover, DHT stabilizes the AR for a longer period [3,4] and exhibits a 5-fold slower dissociation rate from the receptor, than testosterone. Consequently, concentrations of DHT significantly lower than those of the

☆ Proceedings of Xth International Congress on Hormonal Steroids, Quebec, Canada, 17–21 June 1998.

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0960-0760/99 $ - see front matter
PII: S0960-0760(98)00150-2

precursor are required to activate the transcription of androgen-dependent genes. Therefore, the enzyme(s) responsible for DHT formation must be considered important components of the intracellular systems responding to androgens.

Two isoforms of the 5α-R (5α-R type 1 and 5α-R type 2) have been cloned (see Ref. [5] for review). The protein sequences show a modest homology (about 47%), a molecular weight of 28–29 kDa and a high number of hydrophobic amino acid residues, which accounts for the intrinsic membrane localization of both isozymes. Both isoforms of the enzyme 5α-reduce all 3keto, Δ4-steroids, like androgens, progestagens and corticosteroids. However, they possess different kinetics and specificity. The affinity for the type 1 isoform is in the micromolar range for all the substrates, and is much lower than that for the type 2 isoform, which is in the nanomolar range [6]; the conversion yields (V_{max}) detected for the isozymes are much higher for the type 1 isoform. The two isoforms exhibit different pH optima; 5α-R type 1 works well over a wide pH range (from 5 to 8), 5α-R type 2 shows a narrow pH optimum around 5.5, with a very low activity at pH 7.5. 'Selective' blockers of the 5α-R type 2, like finasteride [5], preferential type 2 blockers like suramine (a drug that acts on the NADPH binding sites of the 5α-Rs) [7, 8] and 'specific' inhibitors for the type 1 isoform [9] have already been obtained.

Studies on the tissue- and cell-specific expression of the two isoforms, as well as on the transcriptional control of the two coding genes, are necessary to determine their specific functional roles. Generally, the rat type 1 gene is widely expressed in various tissues, with the highest levels in the liver; on the contrary, the type 2 isoform is mainly concentrated in the androgen-dependent structures, such as the stromal component of the prostate, the epididymis and the seminal vesicles; low levels, if any, have been detected in other tissues [5]. Evidence for different physiological roles of the two isozymes comes also from the observation that a genetically defective type 2 isoform produces in man a syndrome of male pseudohermaphroditism (Imperato–McGinley syndrome); the phenotype is characterized by aplasia of the prostate and ambiguity of the external genitalia [10, 11], the presence of the type 1 isozyme does not seem to be able to replace the inactive type 2 isoform.

The subcellular localization studies performed on the two 5α-Rs indicate that they are strictly associated with cellular membranes [12], but in different cellular compartment; yeast cells genetically transformed to specifically produce each of the two rat isozymes contain the 5α-R type 1 associated with cell nuclei and 5α-R type 2 mainly in the microsomal fractions [12].

A considerable 5α-R activity is present in the CNS, but its functions are poorly understood. This review will summarize recent results obtained in the authors' laboratory, which are indicative of different functional roles of the two isoforms of the 5α-R in the brain.

2. Expression of the two isoforms of 5α-R in the CNS

Several studies indicate that an active 5α-R system is present in the brain. After the gene encoding the type 1 isoform was cloned, the kinetic constants for the activity of the enzyme present in the hypothalamus [13], cerebral cortex, subcortical white matter and purified myelin membranes [14] were shown to resemble those of the recombinant type 1 isoform [12, 15]. The presence of the type 2 enzyme could not be excluded, since its activity could have been masked by the higher conversion capability of the type 1 isoform.

Using reverse transcription-polymerase chain reaction (RT-PCR) technique, it has been demonstrated that the type 1 isoform is expressed in cultures of several brain cell types obtained from the fetal and the perinatal rat CNS (mixed glia, type 1 astrocytes, oligodendrocytes and neurons, which possess the highest levels of expression of the enzyme) [16, 17]. '*In situ*' hybridisation data have also shown the presence of 5α-R type 1 mRNA in selected regions of the brain [18]. The expression of this isoform peaks, at early stages of fetal development (gestational day, GD12), in the proliferating zones close to the ventricular wall; after birth, the mRNA for the 5α-R type 1 is mainly associated with the pyramidal cell layer of the hippocampus, with the subiculum, the cortical plate, the thalamus and the cerebellum. At later time intervals, throughout the adult state, both mRNA and the translated protein of the 5α-R type 1 are found in white matter structures, such as the optic chiasm and the corpus callosum [14, 18–20], where the enzyme is strictly associated with myelin membranes [20]. The K_m values determined in myelin for 5α-R activity (substrate progesterone: male 0.5 μM/female 0.6 μM; substrate testosterone: male 1.1 μM/female 1.5 μM) are all in the micromolar range [21] and are identical to those found for the recombinant 5α-R type 1 [5, 12]. A polyclonal antibody raised against a synthetic hydrophilic peptide deduced from the amino acid sequence of the type 1 5α-R specifically recognises the enzymatic protein in the myelin sheaths of the rat optic nerve [21]. The 5α-R type 1 in myelin membranes probably plays an important protective function, being involved in the catabolism of high levels of neurotoxic steroids (see later).

With regard to the 5α-R type 2 isoform, it has been found that neither the glia nor neurons in culture express the corresponding gene [16]. The absence, in the artificial environment designed for cultured cells, of paracrine, endocrine and/or neuronal regulations

present '*in vivo*' may have modified the physiological situation. In the whole brain of the rat the 5α-R type 1 mRNA is detectable from GD 14 to adulthood; 5α-R type 2 mRNA is also expressed in the brain, but with a totally different profile. This isoform is undetectable at GD14, increases after GD18, peaks at postnatal day (PN) 2 and then decreases gradually to low levels in adults [22]. This pattern correlates with that of circulating levels of testosterone, suggesting an androgenic control on the 5α-R type 2 gene promoter. Moreover, the transient expression of the 5α-R type 2 isoform overlaps the 'critical period' of sexual differentiation of the brain toward male patterns, when not only circulating androgens [23] but also AR [24] are elevated in the CNS, indicating that this isoform of 5α-R could be involved in the control of this process. This hypothesis was straightened by the observation that '*in utero*' exposure to the AR antagonist flutamide inhibits the peak of 5α-R type 2 expression normally present in the brain of male embryos at birth time, but is less effective in females.

To prove the contribution of androgens to the control of the 5α-R type 2 gene, we have taken a new approach, taking advantage from the fact that, under standard conditions, cultured hypothalamic neurons do not express 5α-R type 2 mRNA [16]. Using this model we have analyzed the control of the type 2 gene and found that it is highly induced by testosterone; on the contrary, 5α-R type 1 expression remained unchanged during the treatment [22]. The induction of 5α-R type 2 by testosterone is mediated by activation of the AR, because DHT mimics this effect, while estradiol (which in neurons can derive from testosterone, but activates the ER) was unable to induce the gene expression of the 5α-R type 2 gene (Poletti and Negri-Cesi, unpublished).

Only low levels of 5α-R type 2 expression have been found in the whole brain of adult animals, apparently implying that 5α-R type 2 is not involved in the regulation of the sexually mature brain. A further analysis however has indicated that there is a selective pattern of expression of this isoform in a few localised brain areas; in particular, in adulthood, 5α-R type 2 is highly expressed in the hypothalamus and, to a lower extent, in the hippocampus; very low levels of type 2 mRNA are present in the amygdala, olfactory bulb and in the cerebral cortex (Negri-Cesi *et al.*, unpublished). Surprisingly, cultured hippocampal neurons constitutively express 5α-R type 2 mRNA and do not respond to testosterone or flutamide treatments (Poletti and Viviani, unpublished); in this neuronal cell type the regulation of the 5α-R type 2 promoter probably differs from that present in the hypothalamic neurons.

3. Expression of the two isoforms of 5α-R in LHRH-secreting neurons (GT1)

It is known since a long time that both the hypothalamus and the hippocampus are major targets of sex steroid actions. The hypothalamus is particularly rich of intracellular receptors for gonadal steroids and this brain region, through the effector system of the LHRH secreting neurons, is the primary site of the control of the hypothalamic–pituitary–gonadal axis. The feedback regulation of this system may occur through different pathways: indirectly, via neurotransmitters/neuropeptides, or directly, via steroid receptors located within the LHRH synthesising neurons. Receptors for gonadal steroid have not been detected so far '*in vivo*' in the LHRH synthetizing system [25, 26]. However, '*in vitro*' it has been shown that the LHRH-secreting neuronal cell line GT1 [27] possesses high affinity, low capacity binding sites for both estrogens and androgens [28–31]. The expression in this cell line of classical estrogen [29] and androgen [32, 33] receptors has been later confirmed by RT-PCR [29–31]. The recent discovery of a transcriptionally active estrogen receptor β (ERβ), which shows a low homology with the known ER especially in the N-terminal transactivation domain [32–34] and which is highly expressed in the hypothalamus [35] has prompted several authors to reconsider the whole matter; it emerged that the ER responsible for estrogen binding present in GT1 cells apparently belongs to the now called α subtype (the 'classical' receptor) [31].

No detectable aromatase activity is present in the GT1 cells [28], consequently any effect of estradiol, acting on such cells '*in vivo*' must be linked to an endocrine or paracrine mechanism, through the transport of estradiol formed in surrounding neurons or brought by the general circulation.

On the other hand, the GT1 cells 5α-reduce testosterone to DHT, but their activity at neutral pH is much lower than that usually measured in cultures of fetal neurons [28]. The explanation for this apparent discrepancy has been provided by the observation that, in GT1 cells, the 5α-R enzymatic reaction shows a narrow pH optimum around 5.5 [33] (which is typical for the 5α-R type 2) and a K_m for testosterone very similar to that observed for the recombinant type 2 isozyme expressed in yeast [12]; it is noteworthy that this isoform is usually strictly associated to classical androgen-target structures. The androgen-responsive machinery here described in GT1 cells probably represents the site for the intracerebral negative feedback effect of androgens on the hypothalamic–pituitary–gonadal axis; in fact, DHT is a potent, direct suppressor of LHRH gene expression in GT1 cells [36]. Moreover, androgens are necessary during the fetal and/or early neonatal life to 'masculinize' some hypothalamic cen-

ters (e.g. those controlling male-type gonadotropin and GH secretion) [37, 38]. These priming effects are generally thought to be mediated by the aromatization of androgens to estrogens [39]. As already mentioned, the GT1 cells do not possess the enzyme aromatase [28], produce high levels of 5α-R type 2; it is possible that, in our model, DHT might influence the formation of the sex specific neuronal networks around the LHRH-producing cells by controlling neurite outgrowth in the hypothalamus.

4. Other possible roles of 5α-R isoforms in the brain

4.1. Neurosteroidogenesis

Another possible function of the 5α-R's in the brain is the local intracerebral formation of active anxiolytic/anesthetic steroids. The molecular basis for this mechanism resides in the fact that the activity of the enzyme 5α-R in the CNS, like in many peripheral androgen target structures, is coupled to that of a second enzyme, the 3α-hydroxy-steroid dehydrogenase (3α-HSD). The two enzymes regulate the intracellular concentrations of several steroid molecules, producing either activatory or inactivatory agents. The system is highly versatile, in the sense that every ketoΔ4 steroid may be first 5α-reduced and subsequently 3α-hydroxylated. Through this pathway, DHT is further reduced to 5α-androstane-3α,17β-diol (3α-diol); DHP derived from progesterone is metabolized to allopregnanolone (THP); similarly, deoxycorticosterone (DOC) following transformation to dihydroDOC (DHDOC) gives rise to tetrahydroDOC (THDOC). THP and THDOC are two potent regulators of neuronal functions, with marked sleep-inducing and anesthetic/anxiolytic properties [40]. These steroids possessing CNS depressant properties are unable to interact with the 'classical' intracellular receptors for progesterone and the corticoids but act mainly through the $GABA_A$ receptor complex [41, 42]. It is thus possible that the 5α-R isozymes play physiological roles in situations in which progesterone and/or corticoids are elevated. During stress, the increase of corticoids also augments the formation of tetrahydroderivatives acting on the $GABA_A$ receptor and producing a sedative (anxiolytic) effect [43]. Similarly, at time of parturition, when the 5α-R type 2 isoform is highly expressed in the fetal brain and plasma levels of progesterone are extremely high, these may provide the substrate for the production of anesthetic compounds [44]. The 5α-R type 2 may be involved in the state of sedation occasionally seen during pregnancy [44] and may also provide a possible molecular explanation for the altered behavioural responses sometimes seen in women at the end of the menstrual cycle and in the postpartum period (premenstrual syndrome, postpartum depression), when there is a significant decrease in the formation of 5α-reduced metabolites of progesterone [44–46].

4.2. Protective/catabolic role

Finally, an important catabolic role of 5α-R can also be proposed. The 5α-reduction of the A ring is an irreversible process and allows the subsequent 3α- or (3β)-reduction of the 3-oxo group by the 3α-HSD or by the 3β-HSD; while the process of 3α-hydroxylation may give origin to active steroids (see above), 3β-hydroxylation leads to the formation of inactive compounds [20]. The catabolism of high concentrations of potentially neurotoxic steroids (e.g. the glucocorticoids, which may induce apoptotic processes in particular neuronal populations like the hippocampus, may thus be obtained through this pathway.

The 5α-R type 1, which is present at all stages of development [16, 22] and which shows particularly high concentration in the myelin membranes of axons [14, 15, 21] may help to protect neurons from toxic insults, by regulating the types and the amounts of substances reaching the axons; the enzyme metabolises androgens, progesterone and the glucocorticoids only when they reach high concentration [6] thus working as a component of the myelin filter protecting the neurons from excessive levels of harmful steroid hormones.

The protective role of the 5α-R type 1 has been recently demonstrated by Mahendroo and collegues [47], who have produced transgenic mice carrying an inactive form of 5α-R type 1. In these animals [47, 48], a significant decrease in the number of living fetuses has been observed; the high fetal mortality has been correlated to the formation of toxic levels of estradiol, via an increased substrate availability for aromatization, since testosterone is not removed by the process of 5α-reduction. The toxic role of estradiol in this model has been confirmed by the finding that antiestrogens administered to the mothers reversed the effect [47, 48].

5. Conclusions

The two isoforms of the 5α-R appear to exert key regulatory functions on several distinct brain events and play different physiological roles.

The type 1 isoform of 5α-R is constitutively expressed in the CNS throughout life and probably plays an important 'catabolic' (protective) role in the brain; coupled to the enzyme 3α-HSD it may also facilitate the synthesis of hypnotic-anaesthetic steroids like THP and THDOC.

The type 2 isoform of 5α-R is transiently expressed in the perinatal period and '*in vivo*' its expression is controlled by androgens, at least in males. Testosterone induces 5α-R type 2 expression also in cultured hypothalamic neurons. In adulthood the expression of 5α-R type 2 appears to be confined in the hypothalamus and in the hippocampus and is particularly elevated in LHRH-secreting hypothalamic neurons.

In conclusion, the data discussed suggests that 5α-R type 2 might take part in the process which controls the perinatal differentiation of the brain towards male patterns. The peak of 5α-R type 2 expression in the perinatal period also suggests a possible involvement of this isoform in the formation of anxiolytic/anesthetic steroids at the time of parturition.

Acknowledgements

Grants funding TeleThon-Italy (A.096); CT96. 03105.CT04 (special projects ACRO 95.00395.PF39; FATMA 95.00868.PF41; AGING 95.01020.PF40) by MURST and by AIRC.

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