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Transient Expression of the 5α-Reductase Type 2 Isozyme in the Rat Brain in Late Fetal and Early Postnatal Life*

ANGELO POLETTI, PAOLA NEGRI-CESI, MONICA RABUFFETTI, ALESSANDRA COLCIAGO, FABIO CELOTTI, AND LUCIANO MARTINI

Istituto di Endocrinologia, Università di Milano, 20133 Milan, Italy

ABSTRACT

The enzyme 5α-reductase plays a key role on several brain functions controlling the formation of anxiolytic/anesthetic steroids derived from progesterone and deoxycorticosterone, the conversion of testosterone to dihydrotestosterone, and the removal of excess of potentially neurotoxic steroids. Two 5α-reductase isoforms have been cloned: 5α-reductase type 1 is widely distributed in the body, and 5α-reductase type 2 is confined to androgen-dependent structures. In this study, the gene expression of the two 5α-reductase isozymes has been analyzed in fetal, postnatal, and adult rat brains by RT-PCR followed by Southern analysis. 5α-Reductase type 1 messenger RNA is always detectable in the rat brain [from gestational day 14 (GD14) to adulthood]. 5α-Reductase type 2 messenger RNA expression is undetectable on GD14, increases after GD18, peaks on postnatal day 2, then decreases gradually, becoming low in adulthood. This pattern of expression appears to be correlated with the rate of production of testosterone by the testis. The possible control by androgens of gene expression of the two isozymes has been studied in brain tissues of animals exposed *in utero* to the androgen antagonist flutamide; the sex of the animals was determined by genetic sex screening of the SRY gene located on the Y-chromosome. In the brain of male embryos, flutamide treatment inhibited the expression of 5α-reductase type 2; this effect was much less pronounced in females. Moreover, 5α-reductase type 2 gene expression in cultured hypothalamic neurons is highly induced by testosterone and by the phorbol ester 12-*O*-tetradecanoyl-phorbol-13 acetate. The transient, androgen-regulated, expression of 5α-reductase type 2 overlaps the critical period of development, which may be important for sexual differentiation of the brain and for the formation of anxiolytic/anesthetic steroids involved in the stress responses associated with parturition. (*Endocrinology* **139:** 2171–2178, 1998)

IN MAMMALS, gonadal steroids play an essential role in the process of brain differentiation, especially during embryonic development and in early postnatal life. In particular, testosterone is responsible for the sexual differentiation of the male brain; this includes the organization of both endocrine and behavioral functions, for instance, the dimorphic regulation of gonadotropin and GH secretion, the control of sexual and aggressive behavior, *etc.* This process is thought to be largely mediated by the formation of estrogens by the enzyme aromatase (for review, see Refs. 1 and 2). Dihydrotestosterone (DHT), which is formed in the brain via the 5α-reduction of testosterone, has also been shown to exert specific organizational roles on selected neuronal populations (3–5) and, therefore, is possibly involved in the processes of sexual differentiation of some brain regions.

The physiological importance of 5α-reductase (5α-R) in the brain resides also in its capability to convert progesterone and the corticoids to their respective 5α-reduced derivatives, providing the substrates for the 3α-hydroxysteroid dehydrogenase, the enzyme responsible for the formation of 3α-hydroxy-5α-pregnan-20-one (allopregnanolone or tetrahydroprogesterone) and of 3α,21-dihydroxy-5α-pregnan-20-one (tetrahydrodeoxycorticosterone), which are two potent neurally active steroids. These two 5α-reduced and 3α-hydroxylated compounds are unable to interact with the classical intracellular receptors for progesterone and the glucocorticoids, and their actions are mediated by the γ-aminobutyric $acid_A$ receptor (6). The activation of this receptor may explain their potent anesthetic/anxiolytic activities.

5α-R, then, appears to be a key regulatory enzyme that controls several distinct brain functions; moreover, in cooperation with other enzymatic systems, it may contribute to the catabolism of potentially neurotoxic steroids (*e.g.* the glucocorticoids, which, when present in excess, might induce apoptotic processes in selected neuronal populations of the hippocampus) (7, 8).

Two isoforms of the enzyme 5α-R have been cloned; they catalyze the same reaction, but possess different biochemical and pharmacological properties, distinct cell- and tissue-specific patterns of expression (9, 10), and different subcellular distributions (11, 12). Therefore, the two isozymes may play distinct physiological functions. This hypothesis is supported by the observation that although the type 1 isoform is widely expressed in various tissues (with the highest levels in the liver), the type 2 isoform appears to be selectively concentrated in classical androgen-dependent structures, such as prostate, seminal vesicles, *etc.* (13).

Considerable 5α-R activity has been measured in the central nervous system (CNS) of several mammalian species (14,

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Address all correspondence and requests for reprints to: Angelo Poletti, Ph.D., Istituto di Endocrinologia, via Balzaretti 9, 20133 Milan, Italy. E-mail: angelo.poletti@unimi.it.
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15). The enzymatic activity is more pronounced in brain regions such as the hypothalamus, the midbrain, and all structures rich in white matter; this phenomenon is due to the peculiar localization of elevated levels of 5α-R activity in myelin (14). The type 1 isoform of 5α-R appears to be responsible for the activity in myelin, because the kinetic constants of the enzyme present in the purified myelin are very similar to those found in the recombinant type 1 isozyme (15–18); moreover, a polyclonal antibody raised against the 5α-R type 1 recognizes the enzymatic protein in the myelin sheaths of the axons of rat optic chiasm (18).

Few data are available on the presence and distribution of the two 5α-R isoforms in the rat brain, the developmental patterns of expression, or the possible control mechanisms exerted by hormonal steroids. Specific messenger RNAs (mRNAs) coding for the type 1 isoform (13, 19, 20) have been detected on total RNAs obtained from the whole brain of 7-week-old male rats. The presence of 5α-R type 2 mRNA is still controversial (13, 20). A recent study performed in this laboratory (19) has shown the presence of type 1, but not 5α-R type 2, mRNA in cultured rat brain cells, with maximal levels of expression in neurons. However, cultured cells may not represent the physiological situation occurring *in vivo*, because of the absence of paracrine, endocrine, and/or neuronal regulations.

The aim of the present study was to evaluate, using the RT-PCR technique, the expression of the two isoforms of the 5α-R in rat brain at different stages of development and to clarify whether their expression might be influenced by androgens. This aspect has been studied both *in vivo*, by the *in utero* exposure to the androgen receptor (AR) antagonist flutamide, and *in vitro* on cultured fetal hypothalamic neurons. The brains of male and female fetuses, identified by genetic screening of a marker gene (SRY) of the Y-chromosome, have been separately studied in the experiments with flutamide.

Materials and Methods

Materials

Oligonucleotide sequences: All the synthetic oligonucleotides were obtained from Pharmacia Biotech (Uppsala, Sweden). The following materials were also used: DMEM, Dulbecco's PBS, and trypsin (Biochrom, Berlin, Germany); heat-inactivated FCS (Life Technologies, Grand Island, NY); penicillin, streptomycin, bovine insulin, bovine transferrin, putrescine, sodium selenite, phenol red-free DMEM, diaminobenzidine tetrahydrochloride, steroids, Harris hematoxylin, and chemicals (Sigma Chemical Co., St. Louis, MO); GeneAmp kit (Perkin-Elmer, Palo Alto, CA); and Zeta-Probe (Bio-Rad, Richmond, CA).

Animals

To perform the ontogenetic studies, brains were obtained from rat embryos on gestational days (GD) 14, 16, and 18; on postnatal days (PN) 2, 7, 10, 14, 18, 22, and 28; and in adulthood. The animals were obtained from timed pregnant Sprague-Dawley rats (Charles River, Calco, Italy) that were maintained in animal quarters with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on at 0630 h). The mothers were fed a standard pellet diet and water was provided *ad libitum*. In the experiments performed to evaluate the role of androgens on 5α-R expression, flutamide was injected daily at a dose of 100 mg/kg to timed pregnant rats from GD14 to GD21. The embryos were obtained by surgical procedure on GD21, the presumed day of birth.

Cell cultures

Hypothalamic neuronal cell cultures were obtained from the hypothalamic region of the brain of 16-day-old embryos by disrupting the tissue in phenol red-free DMEM containing 20% FCS. The cell suspension was then plated for 1 h in 10-mm petri dishes to remove fibroblasts (which rapidly adhere to the plate), and floating neurons were collected and plated on polylysine-coated 35-mm petri dishes in phenol red-free DMEM containing 20% FCS. After 24 h, the medium was replaced with phenol red-free DMEM containing 50 U/ml penicillin, 50 U/ml streptomycin, 5 μg/ml bovine insulin, 100 μg/ml bovine transferrin, 100 μM putrescine, and 20 nM sodium selenite until the collection of cells (5 days *in vitro*).

Immunocytochemistry

The immunocytochemical characterization of hypothalamic neuronal cultures was performed using a rabbit polyclonal antibody raised against the neuron-specific enolase (Dako Corp., Milano, Italy). The neuronal cell layer was fixed in 4% paraformaldehyde for 5 min at room temperature. The cells were washed with 0.1% Triton X-100 in phosphate buffer (0.01 M) for 20 min and then incubated with 1.5% normal goat serum for 20 min to block nonspecific binding of the biotinylated secondary antibody. The antibody was applied to the cells at a dilution of 1:10 for 1 h at room temperature. The cells were then washed with 0.1% Triton X-100 in phosphate buffer (0.01 M) for 20 min, incubated with the biotinylated secondary antibody (1:200 dilution; rabbit antirat IgG; Vector Laboratories, Burlingame, CA) for 30 min, then washed three times with 0.1% Triton X-100 in phosphate buffer (0.01 M) for 3 min each time, and incubated with peroxidase-avidin for 30 min. After three washes with 0.1% Triton X-100 in phosphate buffer (0.01 M) for 3 min each time, the cells were finally exposed to the substrate solution (1.6 mg/ml urea hydrogen peroxide and 0.7 mg/ml 3,3′-diaminobenzidine tetrahydrochloride) for 5 min, washed three times with 0.1% Triton X-100 in phosphate buffer (0.01 M) for 3 min each time, stained with Harris hematoxylin, and observed under a light microscope.

RT-PCR

The brain tissues or the cultured hypothalamic neurons were solubilized in guanidium isothiocyanate, and total RNA was prepared by centrifugation over a 5.7-M cesium chloride step cushion as previously described (19). In the experiments requiring genetic sex screening, total genomic DNA was recovered from the intermediate layer of the cesium chloride step cushion of each tube using a sterile syringe, precipitated with isopropanol, washed with 70% ethanol, and then resuspended in sterile water.

RT-PCR was performed using a GeneAmp kit with 2 μg total RNA from each sample. The same extracts of total RNA were subjected to specific analysis for the presence of 5α-R type 1 and type 2 mRNA. Samples of total RNA obtained from adult rat prostate, known to contain high levels of mRNAs of the two isozymes, were used as positive controls (10). The synthetic oligonucleotides used as primers are schematically reported in Fig. 1, and the nucleotide sequences were the following: for 5α-R type 1 amplification: upstream primer, 5′-CGA CCT GCC TGG TTC ATA CA; downstream primer, 5′-GGT CAC CCA GTC TTC AGC AT; and for 5α-R type 2 amplification: upstream primer, 5′-GTC CTG CTG GCT CTC TTC TC; downstream primer, 5′-CAG GCT TCC TGA GCT GGC GC. Nonspecific genomic DNA amplification is avoided in the two types of analyses performed because the sets of primers chosen are located on different exons of each gene (as determined by comparison of the rat and human sequences alignments). Moreover, the RNA preparations were assayed in preliminary experiments in which RT-PCR was performed, omitting the addition of reverse transcriptase to the samples. No specific bands were observed, indicating that genomic DNA could not be amplified.

The RT conditions were 42 C for 45 min followed by 5 min at 95 C, using a final concentration of 1 mM of each deoxy-NTP, 1 U ribonuclease inhibitor, 2.5 U murine leukemia virus reverse transcriptase, and the downstream primer in a final volume of 20 μl. The same buffer (50 mM KCl, 10 mM Tris-HCl, and 2 mM $MgCl_2$) was used for both transcription and amplification. The PCR reaction was performed using the amplification mixture comprised of 2.5 U Ampli-Taq polymerase and the

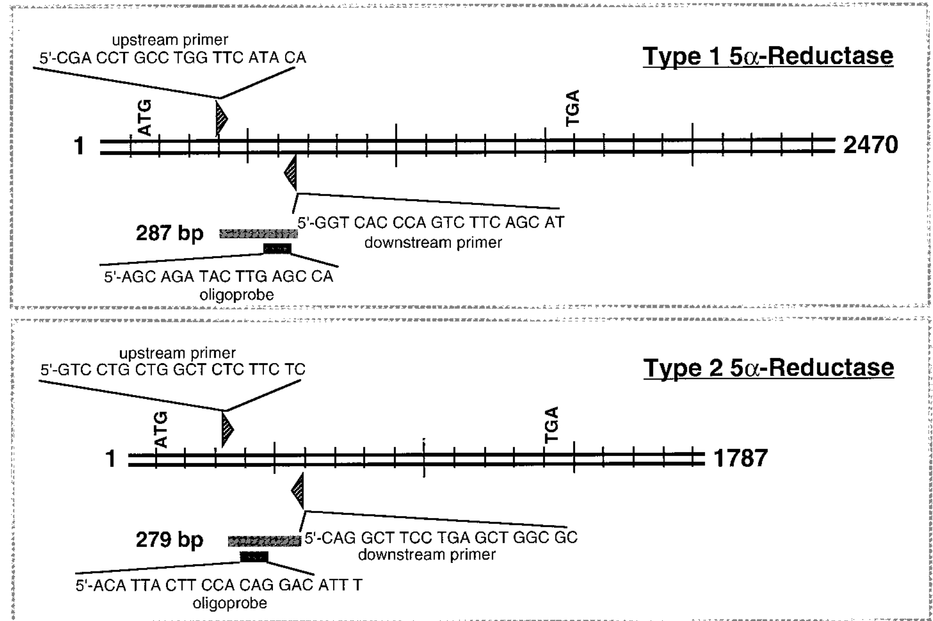

FIG. 1. Location on the 5α-reductase isoform cDNAs of the oligoprimers and oligoprobes used in the RT-PCR analyses of 5α-R type 1 and type 2 gene expression in the rat brain. The nucleotide sequences have been deduced from the published cDNA of the two isoforms and are expected to produce two fragments of about 300 bp; the selection was made in a portion of cDNA nonconserved in the two isoforms and with a limited degree of homology with other published sequences. The primers are expected to be located on different exons of each gene (as determined by comparison of the rat and human sequence alignments); this has been confirmed by the absence of amplified products in PCR analysis performed on genomic DNA (or total RNA), assayed by omitting the reverse transcriptase enzyme in the samples.

upstream primer in a final volume of 100 μl. Samples were amplified by 35 repeated cycles as follows: 95 C for 1 min, 42 C for 1 min, and 72 C for 1 min.

The genetic sex-screening determination, performed on genomic DNA by amplification of the SRY gene located on the Y-chromosome, was obtained using the following primers: upstream primer, 5′-TAC AGC CTG AGG ACA TAT TA; and downstream primer, 5′-GCA CTT TAA CCC TTC GAT GA. The PCR conditions are the same as those described for 5α-R type 1 and type 2 analysis.

The amplification products were separated by electrophoresis on 2% agarose gel and identified by ethidium bromide staining. The amplified complementary DNAs (cDNAs) were transferred to blotting nylon membrane (Zeta-Probe) by capillary elution in 10 × SSC (standard saline citrate) and fixed by baking at 80 C for 2 h under vacuum. Southern analysis was performed using the following synthetic oligonucleotides as radiolabeled probes: 5α-R type 1 oligoprobe, 5′-AGC AGA TAC TTG AGC CA; and 5α-R type 2 oligoprobe, 5′-ACA TTA CTT CCA CAG GAC ATT T. The labeling reactions were performed on the free 5′-end of the oligoprimers using T4 DNA polynucleotide kinase, and [γ-^{32}P]deoxy-ATP. The cDNA on the membrane was then incubated at 45 C for 4 h with the prehybridizing solution, added to the 5′-end ^{32}P-labeled oligonucleotide probes (1×10^6 cpm/ml) specific for the two isoforms of the 5α-R cDNAs, and hybridized at 45 C overnight. After washing, the membranes were exposed to x-ray films.

RT-PCR blanks were performed using distilled water and simultaneously subjected to RT-PCR/Southern blotting with the same reagents and conditions as those described above; in none of the experiments performed could a specific signal be obtained, indicating that no contamination of reagents occurred in these studies.

Results

Gene expression of the two 5α-R isoforms in the rat brain was analyzed using RT-PCR combined with Southern analysis

Figure 1 schematically shows the synthetic primers and oligoprobes selected for RT-PCR analysis of 5α-R type 1 and type 2 gene expression in the rat brain. The nucleotide sequences have been deduced from the published cDNA of the two isozymes and are expected to produce two fragments of approximately the same size. Simultaneous RT-PCR amplifications of 5α-R type 1 and type 2 mRNAs were always performed, using the same RNA preparations with a great excess of oligoprimers; the amplification products were then employed in distinct Southern analyses using ^{32}P-labeled oligoprobes. Using this procedure, a semiquantitative analysis was made possible by evaluating the ratio of the mean optic density (MOD) of the radioactive spots obtained for each of the two 5α-R isoforms after Southern analysis (see below).

Ontogenetic studies on the whole rat brain

The first set of experiments was performed to investigate gene expression of the two types of 5α-R in the brain of rats in late fetal/early postnatal life, a crucial period for sexual differentiation toward the male pattern of the rat CNS. The brains of rat embryos at different gestational days (GD14, -16, and -18); those of male rats taken from PN2 to PN28; and those from adult male rats were considered in this study. The Southern analysis performed on the RT-PCR products of amplification obtained using the set of primers specific for 5α-R type 1 is shown in Fig. 2A. Specific amplification products of the expected size (287 bp) were detectable in the rat anterior prostate (used as positive control) and in all brain samples examined. The expression pattern of this isozyme appeared essentially constant throughout the different time intervals considered. The only exception was a small increase in the signal intensity around the time of birth (GD18).

When gene expression of 5α-R type 2 was analyzed in the same brain samples, a totally different pattern of expression was observed (Fig. 2B). No detectable amplification products (279 bp) of the 5α-R type 2 gene were observed at the earliest stages of fetal brain development (GD14 and GD16), whereas a large increase in gene expression of type 2 mRNA was present in the brains of embryos in the late stages of fetal development (GD18) and in early postnatal life (with an apparent maximum on PN2). After birth, the gene expression of this isozyme gradually decreased, becoming relatively low

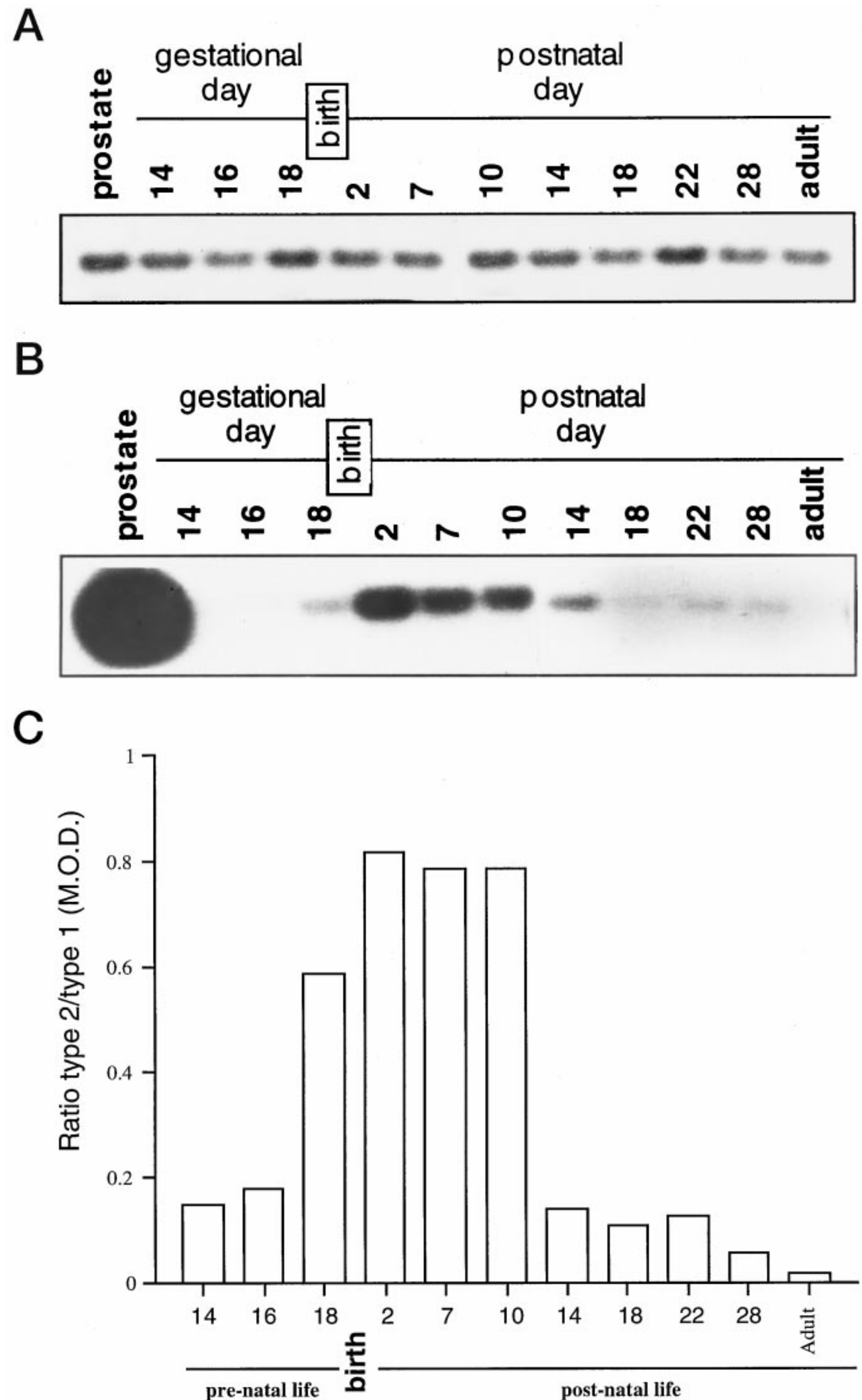

FIG. 2. Ontogenetic expression of 5α-R type 1 and type 2 genes in rat brain. The brains of rat embryos on GD14, 16, and 18; those of newborn male rats taken on PN2, 7, 10, 14, 18, 22, and 28; and those from adult male rats were considered. Total RNA from the rat ventral prostate, a structure known to express both 5α-R isoforms, was used as a positive control. Contemporary RT-PCR amplification of type 1 and type 2 mRNA was performed using the same RNA preparations in the presence of a great excess of oligoprimers; the amplification products were then used in distinct Southern analysis with ^{32}P-labeled oligoprobes. The figure is representative of the data obtained from a single experiment. A, RT-PCR/Southern analysis obtained using the set of primers selective for 5α-R type 1; the specific amplification products appeared at the expected size (287 bp). B, Analysis performed using the set of primers specific for 5α-R type 2 on the same samples reported in A. C, Semiquantitative analysis of the relative expressions of the two isozymes; the plot was obtained by measuring the MOD after SA at the different intervals considered in the analysis described in A and B; the results obtained for 5α-R type 2 analysis were normalized with the values obtained for 5α-R type 1 in the corresponding samples.

after the second week of life and almost disappearing in adulthood. Even if the absolute values of expression of the two isoforms of 5α-R in the brain cannot be determined using RT-PCR, a semiquantitative analysis of the relative expression of the two isozymes was attempted, and the results are shown in Fig. 2C. To this purpose, the MOD values of the radioactive spots obtained on the x-ray films after Southern analysis of 5α-R type 2 mRNA were measured, and these values were normalized with those of 5α-R type 1 measured in the same way in the corresponding samples. It appears that the highest ratio of type 2/type 1 was present during the perinatal period, and this dramatically fell at the beginning of the second week of life. The pattern of expression of the 5α-R type 2 gene appears to be correlated with the level of testosterone produced by the fetal testis, which begins on GD14 and is maximal around the time of birth (21, 22); this suggests a possible involvement of androgens in the control of 5α-R type 2 biosynthesis.

Androgen regulation of 5α-R type 2 gene expression

To verify the hypothesis that endogenous androgens might induce 5α-R type 2 gene expression in the rat brain during the perinatal period, the AR antagonist flutamide was given to pregnant female rats (at daily doses of 100 mg/kg in sesame oil, from GD14 to birth) to counteract the action of testosterone secreted from the testes of male embryos (which normally begins on GD14). Flutamide is known to cross the placental and blood-brain barriers. The experiments were performed using brains at a point when the expression of 5α-R type 2 is maximal, *i.e.* the time of birth (see above). To this purpose, the embryos were surgically removed on the day of presumed birth (21th day in timed pregnant females) to avoid possible delays of delivery consequent to flutamide treatment. As flutamide treatment also blocks the differentiation of secondary reproductive structures, males and females were completely indistinguishable; consequently, a genetic screening of the sex of the embryos was performed. In particular, the presence of the sex-determining gene SRY (An, J., N. Beauchemin, J. Albanese, and A. K. Sullivan, unpublished results; GenBank accession no. X89730), which is located on the Y-chromosome, was analyzed using PCR. Male rats should give specific amplification products of about 300 bp, clearly identifiable by ethidium bromide staining (see Fig. 3, *lower panel*). Figure 3 shows the results of the RT-PCR/Southern analysis performed on the total RNA extracted from the brains of rats exposed *in utero* to flutamide compared with that in brains from unexposed rats. 5α-R type 1 gene expression was very similar in the brains of male and female rats obtained from control and flutamide-treated mothers (Fig. 3, *upper panels*). No sex differences were present in the expression of the type 2 gene between control male and female brains (Fig. 3, *middle panels*). The antiandrogen flutamide significantly decreased the levels of mRNA for 5α-R type 2 in the male rat brain, bringing most of the samples to undetectable levels. Flutamide only slightly diminished the expression of type 2 isoform mRNA in the female brains.

Figure 4 shows a semiquantitative analysis on the relative expressions of the two 5α-R isoforms in the brain of newborn animals, exposed *in utero* to flutamide. A significant decrease in the levels of 5α-R type 2 was evident in the brains of male rats when the MOD (*left panel*) or the 5α-R type 2/5α-R type 1 MOD ratio (*right panel*) was considered. On the contrary, no significant variations were detectable in females.

Control of 5α-R gene expression in hypothalamic neurons

To further clarify the role of androgens in the control of the gene expression of 5α-R type 2, a second approach was used. Cultures of rat fetal hypothalamic neurons obtained from the brain of embryos on GD16 were prepared for these experiments. The hypothalamic neurons were selected because this brain region is one of the primary target structures of androgen action. Immunocytochemical characterization of the cultured hypothalamic cells has been initially performed using a specific monoclonal antibody raised against the neuron-specific enolase. Figure 5 shows that the cultures were mainly composed of neuron-specific enolase-positive cells, possessing a neuronal cell phenotype. These cell preparations were previously shown to express only 5α-R type 1 (19) and consequently were suitable to study a possible induction by testosterone of 5α-R type 2 gene expression. In addition, to verify whether other activators (*i.e.* growth factors, neurotransmitters, neurohormones, *etc.*), interacting at the cell surface and stimulating membrane receptors, might participate in the control of 5α-R genes, the effect of 12-*O*-tetradecanoyl-

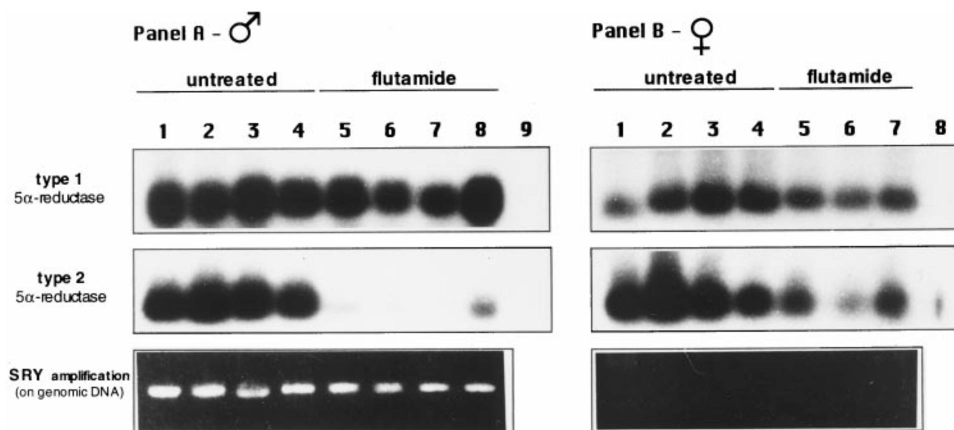

FIG. 3. Effect of *in utero* flutamide exposure on 5α-R type 1 and type 2 gene expression in the rat brain. To assess whether endogenous androgens control the induction of the expression of 5α-R type 2 in the rat brain during the perinatal period, timed pregnant female rats were treated daily with the potent AR antagonist flutamide (at daily doses of 100 mg/kg in sesame oil, from GD14 until birth). The brains of animals were removed on the days of maximal expression of 5α-R type 2, which occurs at the time of birth; therefore, the embryos were obtained by surgical procedure on GD21, which is the presumed day of birth, to avoid possible delay of delivery related to flutamide treatment. A, RT-PCR/Southern analysis of total RNA from male rat brains exposed *in utero* to flutamide compared with those obtained from unexposed rats. *Upper panel*, Analysis of 5α-R type 1 gene expression; *middle panel*, analysis of 5α-R type 2 gene expression; *lower panel*, ethidium bromide staining for the genetic screening of the sex of the embryos, performed by analyzing the presence of the sex-determining gene, SRY, a specific gene localized on the Y-chromosome, by PCR on genomic DNA; male rats give a specific amplification product of about 300 bp. B, RT-PCR/Southern analysis of total RNA from female rat brains exposed *in utero* to flutamide compared with those obtained from unexposed rats. *Upper panel*, Analysis of 5α-R type 1 gene expression; *middle panel*, analysis of 5α-R type 2 gene expression; *lower panel*, ethidium bromide staining for genetic screening of the sex of the embryos, performed by analyzing the presence of the sex-determining gene, SRY, a specific gene localized on the Y-chromosome, by PCR on genomic DNA; female rats do not give specific amplification products of about 300 bp.

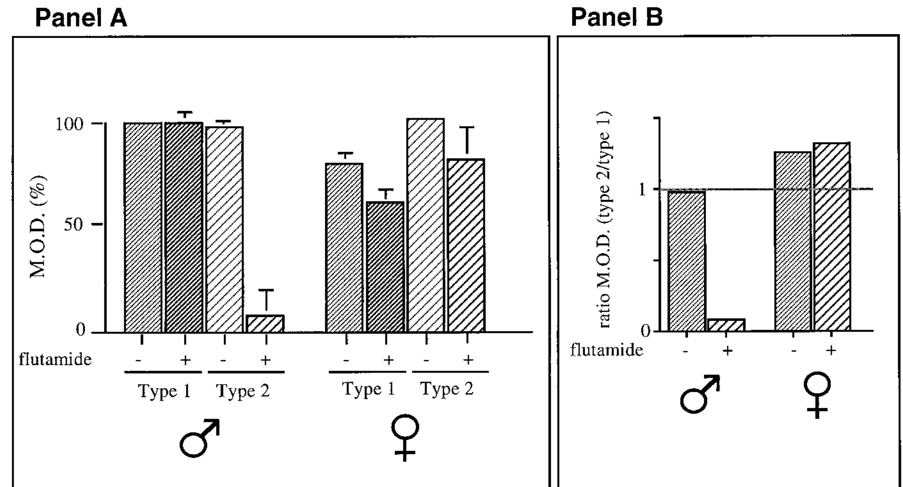

FIG. 4. Analysis of the relative expressions of the two 5α-R isoforms in the brains of newborn animals after exposure *in utero* to flutamide. A, The average ± SEM of the MOD for type 1 or type 2 5α-R gene expression detected by RT-PCR/Southern analysis (and shown in Fig. 3) was evaluated by computer analysis for control or flutamide-treated male and female rats. B, Analysis of the relative expressions of the two isozymes obtained by measuring the MOD after RT-PCR/Southern analysis performed on the samples shown in Fig. 3; the ratio was obtained on the basis of the values shown in A; therefore, the results obtained for 5α-R type 2 analysis were normalized with the values obtained for 5α-R type 1 in the corresponding samples.

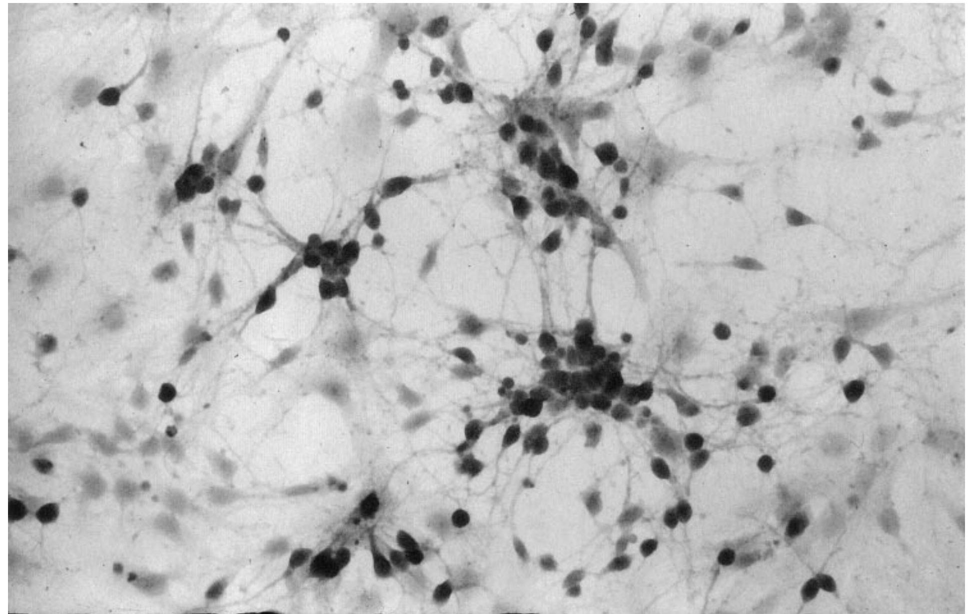

FIG. 5. Immunocytochemical analysis of the hypothalamic neuronal cultures using an antibody raised against the neuron-specific enolase. Cell cultures were obtained from the hypothalamic area of rat embryos on GD16 and maintained as described in *Materials and Methods*. Cells were fixed with 4% paraformaldehyde and incubated with the rabbit polyclonal antibody raised against the neuron-specific enolase at a dilution of 1:10 for 1 h at room temperature. After washing, the cells were incubated with the biotinylated secondary rabbit antirat antibody (1:200 dilution for 30 min at room temperature) and then with peroxidase-avidin (for 30 min at room temperature). After washes, the cells were exposed to the substrate solution (1.6 mg/ml urea hydrogen peroxide and 0.7 mg/ml 3,3′-diaminobenzidine tetrahydrochloride) for 5 min. The cells were then counterstained as described in *Materials and Methods* and observed under a light microscope.

phorbol-13 acetate (TPA), a potent activator of the protein kinase C pathway, was analyzed.

Figure 6 shows the RT-PCR/Southern analysis performed on cultured hypothalamic cells treated with testosterone (20 nM) or TPA (20 nM). No variation in expression levels was observed in the case of 5α-R type 1 mRNA. On the contrary, type 2 mRNA was not detectable in untreated control cells, but appeared to be highly induced after treatment with testosterone. Also, TPA efficiently induced 5α-R type 2 mRNA expression in hypothalamic neurons. In these cells, testosterone may act directly, after conversion into DHT (a processes mediated by the 5α-Rs) and/or into estrogens (formed via the aromatase enzymatic system); both metabolic pathways are present and active in neurons (19). The involvement of the estrogen receptor and estradiol as possible mediators of the action of testosterone on the 5α-R type 2 gene in neurons can probably be excluded, because 5α-R type 2 mRNA was not detectable in neuronal cells cultured in the presence of estrogens (19). Preliminary data obtained in our laboratory indicate that DHT is able to mimic, but with a lower potency, the testosterone-dependent induction of 5α-R type 2 mRNA in cultured hypothalamic neurons.

Discussion

The data presented here have shown that the 5α-R type 1 enzyme is constitutively expressed in the rat CNS at all stages of brain development, with a small increase around the time of birth. This is reminiscent of the increased enzymatic activity measured at neutral pH (optimal for type 1 isozyme) (14) and the type 1 mRNA content that occurs in the rat medial basal hypothalamus on GD18 (20). The expression of 5α-R type 1 is similar in males and females and does not appear to be controlled by androgens or by activation of the protein kinase C pathway.

The gene expression of 5α-R type 2 is totally different. This isoform is transiently expressed in the rat brain in late fetal/early postnatal life. The pattern of expression of 5α-R type 2

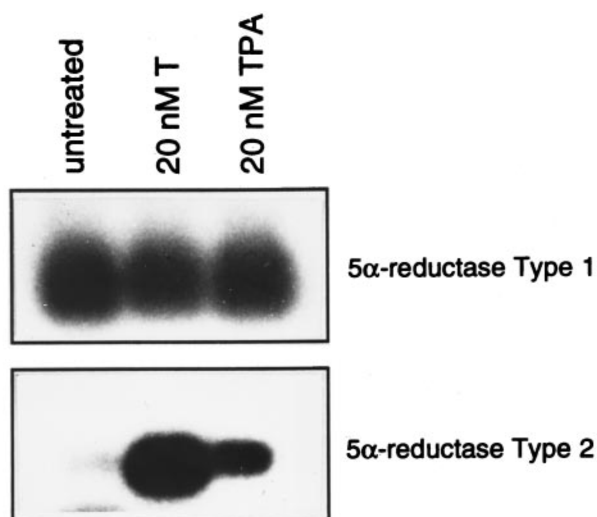

FIG. 6. Control of gene expression of the two isoforms of 5α-R in cultured hypothalamic neurons. Total RNA derived from cultured control and testosterone- or TPA-treated hypothalamic neurons were analyzed with RT-PCR/Southern analysis using oligoprimers specific for type 1 5α-R (*upper panel*) and type 2 5α-R (*lower panel*).

overlaps the secretory profile of testosterone (21, 22), leading us to hypothesize that this isoform could be modulated by the increased levels of circulating androgens occurring in male fetuses around the time of birth; this hypothesis is also supported by the observation that AR (23) is elevated in rat brain during late fetal/early postnatal life and is higher in male than in female brains (23). The role of androgens in the expression of 5α-R type 2 and the possible gender difference were evaluated at the time of its maximal expression in brains using male and female fetuses (identified by genetic sex screening based on the presence of the Y-chromosome) either untreated or exposed *in utero* to the AR antagonist flutamide. In control animals, 5α-R type 2 mRNA levels are similar in the brains of the two sexes. Flutamide treatment produces a significant decrease in the levels of expression of 5α-R type 2 in male animals, whereas it is clearly less effective in modulating the expression of this isoform in females. These data are indicative of a differential mechanism of control of the type 2 isozyme in male and female rat brains. Androgens may then represent the triggering element for the expression of this isoform in males, whereas other control mechanisms are probably involved in females (*e.g.* growth factors, neurotransmitters, other steroids, *etc.*). The androgenic control of 5α-R type 2 gene in the brain has also been confirmed using cultured hypothalamic neurons, but unfortunately it was not possible in this study to separate male and female hypothalamic neurons due to the extremely low recovery of cells per rat hypothalamus.

The data reported here underline the existence of selective ontogenetic patterns of regulation by androgens of the two genes in the brain and generally agree with the data obtained in classic androgen-dependent structures of the urogenital tract. In fact, in those structures, androgens induce 5α-R type 2 mRNA, but not 5α-R type 1 (24). This is peculiar for the perinatal period, as it has been shown that in the prostate of adult rats, both isozymes are controlled by androgens (13, 24).

Our *in vitro* results have also shown that 5α-R type 2 may be induced by activation of the protein kinase C pathway; therefore, not only androgens, but other factors may be of importance in the control of the type 2 gene in the brain. The level of complexity of such regulation requires further investigation, taking into consideration other intracellular signaling pathways.

Together, these data suggest that 5α-R type 2 may be involved in the control of brain sex differentiation occurring during a very critical period, when androgen-organizing effects are thought to take place in the CNS. In principle, testosterone may exert its actions as such or after being metabolized into estrogens or DHT. Although it is generally accepted that the aromatization of testosterone into estrogens is responsible for the sexual differentiation of the brain toward the male pattern, DHT has been recently shown to be essential for the development and organization of selected neuronal populations. For instance, DHT has been shown to induce, in the male rat, a decrease in the volume of the sexually dimorphic nucleus of the accessory olfactory tract to levels found in females (25). Conversely, the postnatal administration of the antiandrogen cyproterone acetate to females produces an increment in the dimensions of this nucleus to volumes not dissimilar from those found in control males. Moreover, in male rats, DHT is needed, together with estrogens, to achieve full masculine development of the sexually dimorphic spinal nucleus of the bulbocavernosus (4). In some amphibians, DHT is responsible for maintenance of the sexual dimorphism of the pretrigeminal nucleus producing vasotocin (26). DHT is detectable in relatively high concentrations in several regions of the rat brain, such as the amygdala and the hypothalamus (27), which are also particularly rich in AR (28). Testosterone and DHT modify the number of branching points in cultured preoptic neurons (4, 5), acting as morphogenetic signals for developing hypothalamic neurons containing the aromatase. Finally, androgens may influence the plasticity and the synaptic connectivity of hypothalamic aromatase-positive neurons (29); these effects are AR mediated, because they are suppressed by the antiandrogen flutamide, but not by the antiestrogen tamoxifen (29). These observations suggest that androgens, at least in these structures, may act through DHT activation of AR. It is the authors' opinion that the role of DHT in brain differentiation has not yet been fully elucidated, possibly because its formation in the CNS is transient, at least for the amounts contributed by the high affinity type 2 isozyme, and is probably limited to some specific CNS structures. It is conceivable that testosterone acts as differentiating agent via estrogens in some brain areas, nuclei, or even single neurons and via the formation of DHT in others.

The low expression observed for 5α-R type 2 in the whole brain of adult animals apparently contrasts with the expression of this isoform in early postnatal life, indicating that this isozyme in adulthood may be expressed in a few localized brain areas; preliminary data obtained in our laboratory have indicated that in adult rats, 5α-R type 2 is selectively expressed in the hypothalamus; after acute stress, this isoform also appears to be induced in the hippocampus. Therefore, the action of 5α-R type 2 might be crucial for the local intracerebral formation of active anxiolytic/anesthetic steroids,

which originate from the 3α-hydroxylation of 5α-reduced derivatives of progesterone and some corticoids (30) that might be involved in modulating stress responses by their interaction with the γ-aminobutyric $acid_A$ receptor complex (6, 31). At time of birth, when the 5α-R type 2 isoform is highly expressed in the brain of fetuses, plasma levels of progesterone are extremely high, providing the substrate for the production of 5α-reduced, 3α-hydroxylated anesthetic compounds, such as tetrahydroprogesterone [also found more concentrated in the brain (see Ref. 6 for details) at the time of parturition]; these two factors (maximal 5α-R type 2 expression and progesterone levels) may be responsible for attenuating the stress of parturition in newborn animals. Again, the increased levels of 5α-R type 2 might be responsible for the sedative state that is occasionally present in the female during pregnancy (6), providing a possible molecular explanation for those phenomena. Conversely, the decrease in the formation of 5α-reduced metabolites that occurs postpartum and at the end of the luteal phase of the menstrual cycle may contribute to the symptoms of postpartum depression and the premenstrual syndrome (32–34).

With regard to 5α-R type 1, this isozyme is constantly present at all stages of development. These data combined with the observation that 5α-R type 1 actively metabolizes androgens, progesterone, and the corticoids only when they reach high concentrations inside the cells (because of the low affinity for the substrates, which is in the micromolar range) (13) lead us to hypothesize that this isoform might play essentially a catabolic function, protecting neurons from the excess of harmful levels of steroid hormones. It is indeed known that corticoids are potent mediators of several apoptotic processes occurring on selected neurons of the hippocampus (7, 8, 23, 35). Moreover, in the rat brain, 5α-R type 1 is expressed in oligodendrocytes (19), and the enzymatic protein is highly concentrated in the myelin membranes of axons (17, 18) formed by these cells. It is well known that myelin sheaths help protect neurons from toxic insults, regulating the types and amounts of substances reaching the axons; 5α-R type 1 may thus work as a component of the myelin filter. Finally, another protective role for 5α-R type 1 has been recently demonstrated in transgenic mice carrying a mutated inactive form of 5α-R type 1 (36, 37). These animals have a smaller litter size due to a higher fetal mortality during gestation caused by an excess estradiol levels, derived from increased testosterone bioavailability for aromatase, not removed by 5α-reduction (37).

The 5α-R isozymes may then play different key regulatory roles in the CNS, on one side by controlling several specific brain functions and on the other side by participating in the catabolism of high concentrations of potentially neurotoxic steroids.

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