Journal of Neurochemistry
Lippincott-Raven Publishers, Philadelphia

Expression of Androgen-Activating Enzymes in Cultured Cells of Developing Rat Brain

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Abstract: Dihydrotestosterone and estradiol, two active metabolites formed locally in the brain from testosterone, modulate several functions of the developing rat CNS; these compounds derive from the 5α-reduction or the aromatization of the A-ring of the hormone. Also, progesterone and corticosteroids may be 5α-reduced and subsequently 3α-hydroxylated, becoming modulators of specific neuronal functions. Although the aromatase is a single enzyme, two types of 5α-reductase have been cloned, showing peculiar biochemical properties and probably different functions. Therefore, the isoform(s) of the enzyme 5α-reductase(s) present in early stage of brain development have been characterized in primary neuronal and glial cell cultures obtained from the fetal or neonatal rat brain, respectively. Aromatase expression was also studied. The results have shown that in all the brain cells examined type 1 5α-reductase mRNA is expressed. No specific transcript of type 2 5α-reductase is detectable in any of the cell types examined. Finally, the aromatase gene is expressed only in cultured fetal neurons and especially in those derived from the hypothalamic area of the rat embryos. It is interesting that no aromatase mRNA is detectable in mixed glia or in type 1 astrocytes and oligodendrocytes cultured separately. **Key Words:** Androgens — 5α - Reductases — Aromatase — Neurons — Glial cells—Rat brain.
J. Neurochem. **68,** 1298–1303 (1997).

Hormonal steroids are known to modulate several CNS functions. For instance, androgens appear to be responsible for the control of the sexual differentiation of the brain as well as of adult male sexual behavior; progesterone and deoxycorticosterone, in high doses, may exert anxiolytic and anesthetic properties (Paul and Purdy, 1992). It has been demonstrated that some of these actions may be mediated by steroid metabolites, rather than by the hormones themselves. The sexual differentiation of the brain is linked to the aromatization of testosterone to estrogens; the anxiolytic/anesthetic properties of progesterone and of deoxycorticosterone are due to their respective 5α-reduced-3α hydroxylated metabolites, 3α-hydroxy, 5α-pregnan-20-one (allopregnanolone or tetrahydroprogesterone), and 3α,21-dihydroxy-5α-pregnan-20-one (tetrahydrodeoxycorticosterone) (Negri-Cesi et al., 1996). These compounds do not bind to classic steroid receptors but interact with the modulatory subunits of the GABA-gated Cl^- channel of the $GABA_A$ receptor (Majewska et al., 1986; Gee et al., 1988; Majewska, 1992). Therefore, it is presently believed that the enzymatic complexes responsible for these transformations, respectively, aromatase (Aro) and 5α-reductase (5α-R) 3α-hydroxysteroid dehydrogenase system, play significant roles in regulating brain functions.

5α-R reduces the double bond in the 4–5 position of the A-ring of several 3-keto-Δ^4 steroids, e.g., testosterone, progesterone, deoxycorticosterone, corticosterone, etc., and is widely distributed in the rat brain (Celotti et al., 1992). 5α-R activity has been detected in several brain regions and structures and is particularly high in the white matter and in the myelin (Celotti et al., 1992). Moreover, among the various cell types present in the CNS, this enzymatic activity is much higher in neurons than in glial cells (oligodendrocytes and astrocytes) (Melcangi et al., 1993). Recently, two different isoforms of 5α-R have been cloned (Russell and Wilson, 1994) and found to possess a limited degree of homology (44%) as well as different biochemical properties: the pH optima are neutral to alkaline for the type 1 and acidic (pH 5.5) for the type 2 isoform, and the affinity for the various substrates (testosterone, progesterone, corticosterone, etc.) is generally lower for the type 1 than for the type 2 isoform. Type 1 and type 2 isozymes also differ in their tissue and cellular distribution (Normington and Russell, 1992), in their subcellular compartmentalization (Poletti et al., 1996; Span et al., 1996), and in their sensitivity to inhibitors (Russell and Wilson, 1994). Because of

Received September 23, 1996; revised manuscript received November 6, 1996; accepted November 6, 1996.

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Abbreviations used: Aro, aromatase; DHT, dihydrotestosterone; 3α-diol, 5α-androstane-3α,17β-diol; div, days in vitro; PCR, polymerase chain reaction; 5α-R, 5α-reductase; RT, reverse transcription.

these differences, it is expected that the two 5α-R isozymes, even if they catalyze the same reaction, might exert different physiological functions. Moreover, it is interesting that the gene expression of the type 1 5α-R is heterogeneous and may give rise to two forms, differing in four amino acids in the N-terminal portion of the molecule; the two subforms of the 5α-R type 1 possess different affinity for the substrates (Lopez-Solache et al., 1996).

Few data are available on the distribution of the two 5α-R isozymes in the rat brain. Using in situ hybridization techniques, the presence of the mRNA for the type 1 5α-R has been demonstrated in specific areas of the rat brain, with three distinct patterns of expression: during early fetal development type 1 mRNA is expressed in the proliferating region close to the ventricular wall of the CNS; during late fetal/early postnatal development, the expression in the ventricular zone decreases, whereas an increase of type 1 5α-R expression is observed in the cortical plate and in the thalamus; and finally, in adult life, type 1 5α-R expression appears to be confined to white matter structures (Lauber and Lichtensteiger, 1996). These results agree with the distribution of the activity of the enzyme found in the rat brain (Poletti et al., 1990; Celotti et al., 1992) and also with the distribution of the protein analyzed by immunohistochemistry (Tsuruo et al., 1996). It is important to note that another recent immunohistochemical study has shown the presence of the type 1 isozyme in glial cells, and not in neurons (Pelletier et al., 1994), in apparent conflict with the fact already mentioned that enzymatic activity is highest in neurons (Melcangi et al., 1993). This could suggest that the enzymatic activity found in neurons might be the type 2 isoform; however, the expression of the type 2 5α-R in the rat brain is still controversial. Northern analysis performed on total RNA obtained from the whole brain of 7-week-old male rats has suggested that the CNS expresses both forms of the enzyme, with a great predominance of the type 1 isoform (Normington and Russell, 1992). More recently, using the same technique, the presence of only type 1 mRNA has been described in the brain of male rats of the same age (Lephart, 1993); it must be recalled, however, that northern analysis is probably not sensitive enough to exclude the presence of type 2 mRNA and consequently of the enzymatic protein.

The second androgen-activating enzyme, Aro, appears to be a single enzyme, with the same amino acid sequence in the different tissues in which it is present, even if its expression appears to be under the control of tissue-specific promoters. Studies on the regional distribution of Aro in the brain have indicated that this enzyme has a discrete distribution, particularly in the hypothalamus, the preoptic area, and the limbic system, i.e., in structures involved in the control of reproductive functions. Studies on the localization of the enzyme, performed by assaying the enzymatic activity in primary cultures of different brain cells (Negri-Cesi et al., 1992) and by immunohistochemical (Shinoda, 1994) or by in situ hybridization (Lauber and Lichtensteiger, 1994) techniques on the total rat brain, agree in indicating that only neurons aromatize androgens; however, the presence of a small amount of Aro or of its expression in other CNS cell types has not been carefully evaluated using the highly sensitive techniques presently available.

The aim of the present study was (a) to characterize the pH optima of the 5α-R(s) present in primary cell cultures of neurons and mixed glia obtained from the fetal or neonatal rat brain, to obtain a possible indication on the enzyme isoform responsible for such conversion, and (b) to analyze the expression of the two types of 5α-Rs and of Aro in the different cell types of the CNS, using the most powerful technique presently available, i.e., reverse transcription (RT)–polymerase chain reaction (PCR).

MATERIALS AND METHODS

Animals

The embryos and the newborn rats were obtained from Sprague–Dawley mothers (Charles River, Italy) that were maintained in animal quarters with controlled temperature and humidity. The light schedule was 14 h of light and 10 h of dark (lights on at 0630 h). The mothers were fed a standard pellet diet, and water was provided ad libitum.

Cell cultures

Neuronal cells were cultured from total brain of 15-day-old embryos obtained from time-pregnant mothers (day 0 = sperm-positive smears) as previously described (Melcangi et al., 1993) and were used at 6 days in vitro (div); hypothalamic neuronal cell cultures were obtained from the hypothalamic region of the brain of 16-day-old embryos by dissolving the tissue in Dulbecco's modified Eagle's medium. The total cells in suspension were then plated for 1 h in 10-mm-diameter Petri dishes to remove fibroblast cells (which adhere rapidly to the plate), and the floating neurons were collected and plated on polylysine-coated 35-mm-diameter Petri dishes in phenol red-free Dulbecco's modified Eagle's medium containing 20% fetal calf serum. After 24 h the medium was replaced with a chemically defined medium (Dulbecco's modified Eagle's medium without phenol red containing 50 U/ml penicillin, 50 U/ml streptomycin, 5 μg/ml bovine insulin, 100 μg/ml bovine transferrin, 100 μ*M* putrescine, and 20 n*M* sodium selenite) until the collection of the cells (5 days div). Mixed glial cell cultures were obtained from 1- or 2-day-old newborn rats and were used at 19 div (Melcangi et al., 1993). Type 1 astrocyte cultures were obtained from mixed glial cultures (at 14 days div) by shaking the flasks overnight at 37°C and replating the cells from the bed layer according to the methods of McCarthy and DeVellis (1980); the astrocytes were used after 5 div. Oligodendrocyte cultures were obtained from primary cultures of mixed glia [5 div (Besnard et al., 1989)] and were used at 6 div (Melcangi et al., 1993).

Assay of the 5α-R activity

To determine the pH optimum of the enzymatic isoform(s) present in the cultured brain cells, the incubations were performed in phosphate-buffered saline solution (250

μl) at various pH values (ranging from 4 to 8) in the presence of an NADPH generating system (Poletti et al., 1990) and 3×10^{-6} *M* [^{14}C]testosterone (specific activity, ~56.9 mCi/mmol; Amersham, U.K.). The incubations were carried out for 2 h at 37°C in a Dubnoff metabolic shaker under a stream of O_2/CO_2 (98:2). Vials without tissue provided the blanks for each pH value, and protein content was evaluated according to the method of Bradford (1976).

Detection of metabolites. At the end of the incubation the reaction was stopped by freezing the samples to −20°C. Tritium-labeled dihydrotestosterone (DHT) and 5α-androstane-3α,17β-diol (3α-diol) (~5,000 dpm each) were added to each sample to evaluate the recoveries. The metabolites formed were extracted twice with diethyl ether and separated by TLC using an eluting mixture of dichloromethane/diethyl ether (11:1 vol/vol). The total 5α-R activity was expressed as the sum of the two major testosterone metabolites, DHT and 3α-diol.

RT-PCR analysis

Oligonucleotide sequences. All the synthetic oligonucleotides were deduced from published sequences of the cDNA of the two isoforms of 5α-R and of Aro and obtained from Pharmacia Biotech (Sweden). All the various types of brain cultured cells were solubilized in guanidium isothiocyanate, and total RNA was prepared by centrifugation over a 5.7 *M* cesium chloride step gradient.

RT and PCR were performed using a GeneAmp kit (Perkin Elmer) on 2 μg of total RNA from each sample. The same extracts of total RNA were subjected to specific analysis for the presence of 5α-R1, 5α-R2, and Aro mRNAs. Samples of total RNA obtained from both adult rat abdominal skin and prostate, known to contain high levels of both 5α-R1 and 5α-R2 mRNA (Russell and Wilson, 1994), were used as positive controls for 5α-R isoforms; samples of total RNA extracted from adult rat ovary, known to contain high levels of Aro mRNA, or from rat placenta, which does not express the Aro gene, were used as positive or negative controls, respectively, for the Aro amplification. The synthetic oligonucleotides used as primers for the amplifications are schematically reported in Fig. 1, and the nucleotide sequences were as follows: for 5α-R 1 amplification, upstream primer, 5′-CGA CCT GCC TGG TTC ATA CA; downstream primer, 5′-GGT CAC CCA GTC TTC AGC AT; for 5α-R 2 amplification, upstream primer, 5′-GTC CTG CTG GCT CTC TTC TC; downstream primer, 5′-CAG GCT TCC TGA GCT GGC GC; and for Aro amplification, upstream primer, 5′-TTG TTG TTA AAT ATG ATG CC; downstream primer, 5′-ATA CCA GGT CCT GGC TAC TG (Bulun et al., 1993). Genomic DNA amplification is avoided in the three types of analysis performed because of the properties of the set of primers chosen; those 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 the RT-PCR was performed omitting the addition of the reverse transcriptase in the samples. No specific bands were observed, indicating that genomic DNA could not be amplified.

The RT conditions were as follows: 42°C for 45 min followed by 5 min at 95°C, using a final concentration of 1 m*M* each deoxynucleotide triphosphate, 1 U of RNase inhibitor, 2.5 U of murine leukemia virus reverse transcriptase, and the downstream primer in a final volume of 20 μl. The same buffer (50 m*M* KCl, 10 m*M* Tris-HCl, and 2 m*M* $MgCl_2$) was used for both transcription and amplification. The final amplification mixture comprised 2.5 U of Ampli-Taq polymerase (GeneAmp Kit; Perkin-Elmer) and the upstream primer in a final volume of 100 μl. Samples were amplified by repeated cycles (35) at 95°C for 1 min, 42°C for 1 min, and 72°C for 1 min.

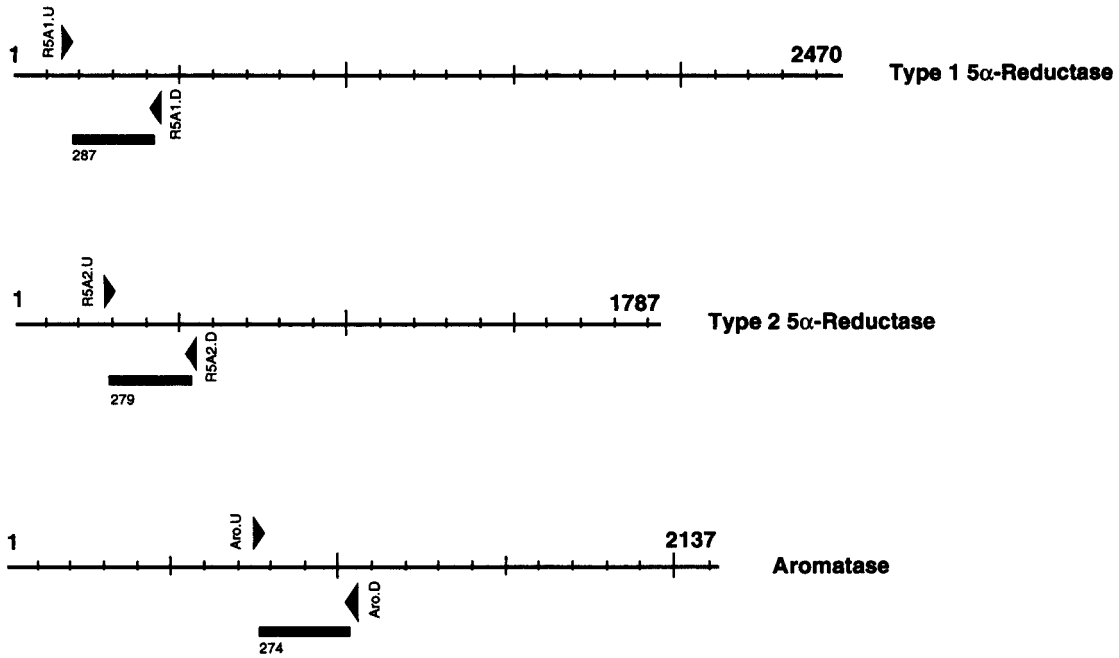

FIG. 1. Schematic representation of the positions of the three sets of oligoprimers, on the corresponding published cDNA, used in RT-PCR of the mRNAs of the two 5α-R isoforms and of Aro mRNAs.

Amplification products were separated by electrophoresis in 2% agarose gel, which was visualized with ethidium bromide staining. The amplified cDNAs were transferred to a blotting membrane (Zeta-Probe; Bio-Rad, Richmond, CA, U.S.A.) by capillary elution in 10× SSC (standard saline citrate) and fixed by baking at 80°C for 2 h under vacuum. The synthetic oligonucleotides used as radiolabeled probes in Southern analysis were as follows: 5α-R 1 oligoprobe, 5′-AGC AGA TAC TTG AGC CA; 5α-R 2 oligoprobe, 5′-ACA TTA CTT CCA CAG GAC ATT T; and Aro oligoprobe, 5′-CAC ATA ATG AAG CAC AAT CAT TA. The labeling reactions were performed on the free 5′ end of the oligoprimers using T4 DNA polynucleotide kinase and [γ-^{32}P]dATP. The cDNA on the membrane was then incubated at 45°C for 4 h with the prehybridizing solution, added to the 5′-^{32}P-end-labeled oligonucleotide probes (1×10^6 cpm/ml) specific for the cDNAs of the two isoforms of 5α-R and of Aro, 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 described. In any of the experiments performed no specific signal could be obtained, indicating that no contamination of any reagents occurred in these studies.

RESULTS AND DISCUSSION

This first set of experiments was designed to dissect out which 5α-R isoform(s) are present in the rat brain, by taking advantage of the different pH optima of the two 5α-R isoforms; the variation of the enzymatic activity of the 5α-R has been analyzed as a function of the pH (from 4 to 8) in lysates of cultured fetal neurons and neonatal mixed glia cells, by measuring the formation of labeled DHT and 3α-diol from [^{14}C]-testosterone. Figure 2 shows the pattern of 5α-R activity in the two types of cell preparations, expressed as the sum of the amounts of the two 5α-R metabolites formed per milligram of protein in 2 h of incubation. The results show that the 5α-R activity present in rat fetal neurons obtained from total brain and in rat neonatal mixed glial cell cultures does not possess a clear-cut pH dependency, being similarly active at pH values ranging from 4 to 8; no peak of activity was obtained for either type of cell culture at acidic pH. Moreover, at all pH values, fetal neurons in culture possess about a twofold higher 5α-R activity than mixed glial cell cultures obtained from newborn rats. These data seem to assign to the type 1 isoform the reducing activity present in the cells examined and confirm a previous finding indicating that neurons possess a 5α-R activity higher than that of glial cells (Celotti et al., 1992; Melcangi et al., 1993). It is noteworthy that the characteristic peak of activity at pH 5.5 of the 5α-R type 2 is absent in both types of cultures; however, the enzymatic activity present at pH 4 and 5 probably cannot be ascribed to the traditional type 1 alone because under these conditions, when the recombinant enzyme is expressed in mammalian cells (Normington and Russell, 1992) or in yeast cells (Poletti et al., 1996), type 1 activity is reduced to 50% or even more. One possibility to explain this activity is to postulate the existence in the rat brain of a subtype of the type 1 5α-R like the one recently described by Lopez-Solache et al. (1996), which is an elongated form (four amino acids in the N-terminal region) derived from the same gene. Another possibility is the hypothesized existence of a third type of 5α-R (Lephart, 1993), which, however, has not been cloned so far.

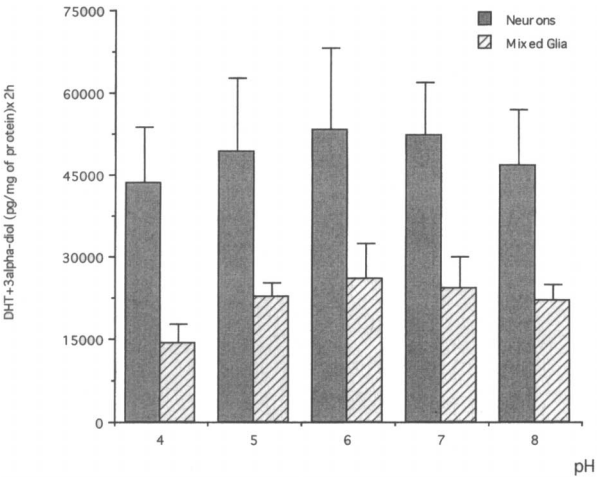

FIG. 2. Variation of the 5α-R activity as a function of pH in cultured rat neurons derived from the brain of embryos at gestational day 15 and rat mixed glial cells derived from the brain of newborn animals. Enzymatic activity was measured as picograms per milligram of protein of labeled 5α-reduced metabolites formed after incubation of [^{14}C]testosterone, in the presence of NADPH as cofactor. See Materials and Methods for details.

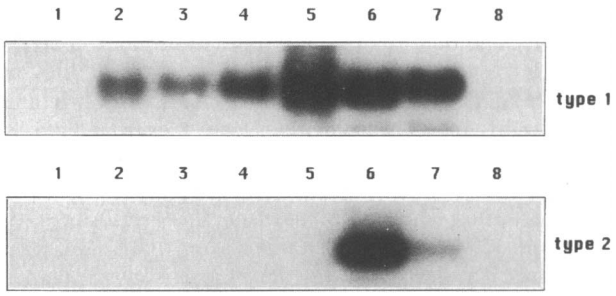

FIG. 3. Southern analysis performed on the RT-PCR product of amplification obtained using specific sets of oligoprimers for the two isoforms of the 5α-R. The amplification was performed on the total RNA derived from the following samples: lane 1, DNA markers; lane 2, mixed glial cells; lane 3, type 1 astrocytes; lane 4, oligodendrocytes; lane 5, neurons derived from the whole brain; lane 6, rat adult ventral prostate; lane 7, rat abdominal skin; and lane 8, water.

Figure 3 shows the autoradiography obtained after Southern analysis on the amplification products of the RT-PCR for type 1 or type 2 5α-R performed on the RNAs of the different cultures of brain cells. A specific transcript of the type 1 5α-R (upper panel) is detectable in RNA samples obtained from the mixed glia

(lane 2), type 1 astrocytes (lane 3), oligodendrocytes (lane 4), and neurons (lane 5); the amplification products correspond in size to those observed in the rat prostate (lane 6) and in the rat abdominal skin (lane 7), used as positive controls. Lane 1 corresponds to the DNA markers, whereas lane 8 corresponds to the blank, in which no total RNA was added. No specific amplification products for the type 2 isoform (Fig. 3, lower panel) have been observed after the RT-PCR of the same samples tested for the type 1 isozyme (lanes 2–5), even after prolonged exposure of the membrane to x-ray film (data not shown). A specific band of hybridization of the expected size (279 bp) is amplified from the mRNAs of the two control tissues (lanes 6 and 7). These data indicate that, in the present experimental conditions, neither glial cells nor neurons are able to express the type 2 5α-R gene. Even if RT-PCR is not a quantitative technique, it is interesting to note that the signal obtained in lane 5 (neurons) is almost two to three times higher (as evaluated by measuring the optic density of the corresponding spots; data not shown) than that obtained for glial cells, suggesting that, among brain cells, the neurons are those expressing the highest levels of type 1 5α-R. These data confirm and extend those previously reported by this laboratory (Celotti et al., 1992; Melcangi et al., 1993), in which a much higher 5α-R activity, as measured using a radioenzymatic procedure, was found in fetal neurons than in any type of neonatal glial cells. Pelletier et al. (1994) have reported results that apparently conflict with those described here. In fact, these authors failed to detect any 5α-R immunoreactivity in the neuronal component of the rat brain using an antibody raised against the human type 1 5α-R; in their study, the material with the highest level of immunoreactivity was diffusely distributed throughout the cytoplasm of glial and ependymal cells. Possible explanations for this discrepancy may reside in several facts. First, the data presented here have been obtained by measuring the actual activity of the enzyme as well as its gene expression; in contrast, Pelletier et al. (1994) used only a morphological approach for "quantifying" the enzymatic protein. Moreover, the observations of Pelletier et al. (1994) were performed using an antibody raised against a synthetic peptide reproducing the sequence of the human type 1 5α-R, which is only partially conserved in the corresponding rat isoform (Normington and Russell, 1992; Pelletier et al., 1994); consequently, the possibility of a nonspecific reaction cannot be excluded, especially because the authors have not provided evidence on the efficiency of the antibody toward the rat isozyme.

The presence of the type 1 5α-R gene expression and functional enzyme in all cells obtained from the fetal or perinatal rat CNS, and reported in the present study, agrees with a recent observation performed using in situ hybridization. In these studies the presence of the mRNA for this isoform in specific regions of the brain at different stages of development (Lauber and Lichtensteiger, 1996) has been analyzed. These data have shown that type 1 5α-R mRNA is already expressed in early stages of fetal development (the first results were recorded on gestational day 12) and is maximal in the proliferating zones close to the ventricular wall. With the increase of the age of the animal, a substantially different localization was observed: the expression of the enzyme is especially elevated in the pyramidal cell layer of the hippocampus, in the subiculum, in the cortical plate, in the thalamus, and in the cerebellum at postnatal day 6. It is interesting that at later intervals until adulthood, the level of the mRNA of the type 1 isoform is in contrast elevated in white matter structures, such as the optic chiasma, the corpus callosum, etc. Also, this result agrees with previous data of our laboratory (Poletti et al., 1990; Celotti et al., 1992). During the late (postnatal) stages of ontogeny, 5α-R enzyme expression appears to parallel the process of myelinization (Celotti et al., 1992).

The expression of Aro in the different cell types of the brain was also investigated using RT-PCR. In these experiments, in addition to neurons of total fetal rat brain (gestational day 15), also those obtained from the hypothalamic region of the fetal rat brain (which are known to possess the highest Aro activity) at gestational day 16 were analyzed. Figure 4 shows the Southern analysis performed on the amplified products obtained with the RT-PCR technique. The rat ovary has been used as a positive control (lane 7), whereas the rat placenta, a tissue devoid of Aro mRNA, has been used as a negative control (lane 8). As shown in Fig. 4, mixed glia (lane 2), type 1 astrocytes (lane 3), and oligodendrocytes (lane 4) do not contain any Aro transcripts. In contrast, in the mRNA samples of total brain neurons, and especially of hypothalamic neurons, a specific amplification product of the expected size was present in correspondence to that obtained from the rat ovary, indicating that the Aro gene is expressed only in neurons and is probably particularly expressed in those derived from the hypothalamus.

The specific localization of Aro in neurons and not

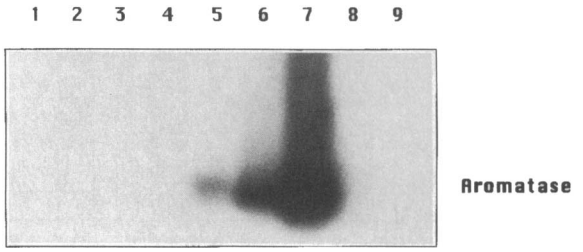

FIG. 4. Southern analysis performed on the RT-PCR product of amplification obtained using specific sets of oligoprimers for Aro. The amplification was performed on the total RNA derived from the following samples: lane 1, DNA markers; lane 2, mixed glial cells; lane 3, type 1 astrocytes; lane 4, oligodendrocytes; lane 5, neurons derived from the whole brain; lane 6, neurons derived from the hypothalamic area; lane 7, rat ovary in estrus; lane 8, rat placenta; and lane 9, water.

in glial cells has been previously described by immunohistochemical (Shinoda, 1994) and in situ hybridization (Lauber and Lichtensteiger, 1994) techniques and, in our laboratory, by a more direct approach, i.e., via the evaluation of the Aro activity in primary cultures of different brain cell populations (neuronal and glial cells) (Negri-Cesi et al., 1992). However, the Aro and its expression were recently found by in situ hybridization analysis, northern blot analysis, and measurement of the enzymatic activity in enriched glial cell cultures of the developing zebra finch telencephalon (Schlinger et al., 1994). It is obvious that a species difference may be responsible for this apparent discrepancy, as the zebra finch is a nonmammalian species.

Acknowledgment: This article is dedicated to the memory of Giuseppe Poletti. This work was supported by CNR funding (Special Projects ACRO grant 95.00395.PF39, FATMA grant 95.00868.PF41, and AGING grant 95.01020.PF40) by MURST and by AIRC.

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