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Expression of Cytochrome P450 Side-Chain Cleavage Enzyme and 3β-Hydroxysteroid Dehydrogenase in the Rat Central Nervous System: A Study by Polymerase Chain Reaction and In Situ Hybridization

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Abstract: In examining steroid synthesis in the CNS, expression of the mRNAs encoding for cytochrome P450 side-chain cleavage enzyme ($P450_{SCC}$) and 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD) has been studied in the rat brain. $P450_{SCC}$ transforms cholesterol into pregnenolone and 3β-HSD transforms pregnenolone into progesterone. PCR was used to amplify cDNA sequences from total RNA extracts. Classical steroidogenic tissues, like adrenal and testis, as well as the non-steroidogenic tissue lung have been used as controls. The expression of $P450_{SCC}$ and 3β-HSD have been demonstrated by PCR in cortex, cerebellum, and spinal cord. In addition, primary cultures of rat cerebellar glial cells and rat cerebellar granule cells were found to express $P450_{SCC}$ and 3β-HSD at comparable levels. Furthermore, three of the four known isoenzymes of 3β-HSD were identified, as determined using selective PCR primers coupled with discriminative restriction enzymes and sequencing analysis of the amplified brain products. Using RNA probes, in situ hybridization indicated that $P450_{SCC}$ and 3β-HSD are expressed throughout the brain at a low level and mainly in white matter. Enrichment of glial cell cultures in oligodendrocytes, however, does not increase the relative abundance of $P450_{SCC}$ and 3β-HSD mRNA detected by PCR. This discrepancy suggests that the developmental state of cultured cells and their intercellular environment may be critical for regulating the expression of these enzymes. These findings support the proposal that the brain apparently has the capacity to synthesize progesterone from cholesterol, through pregnenolone, but that the expression of these enzymes appears to be quite low. Furthermore, the identification of these messages in cerebellar granule cell cultures implies that certain neurons, in addition to glial cells, may express these steroidogenic enzymes. **Key Words:** Neurosteroids—Side-chain cleavage enzyme—3β-Hydroxysteroid dehydrogenase—PCR—In situ hybridization.
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Certain steroids have been shown to alter neuronal excitability through binding to neurotransmitter-gated ion channels (McEwen, 1991; Paul and Purdy, 1992). The best studied of these neuroactive steroids are 3α-hydroxy ring A–reduced metabolites of progesterone and deoxycorticosterone, which interact potently with the $GABA_A$ receptors (Majewska et al., 1986; Morrow et al., 1987; Gee et al., 1988; Puia et al., 1990). In addition, 5α-pregnane steroids like 5α-dihydroprogesterone or 5α-dihydrodeoxycorticosterone may regulate gene expression, via the progesterone receptor, at nanomolar concentrations (Rupprecht et al., 1993). Relevant to the relationship between steroids and neuronal activity, evidence is now accumulating that certain glial cells may be steroidogenic (Hu et al., 1987; Le Gascogne et al., 1987; Jung-Testas et al., 1989; Iwahashi et al., 1990; Guarneri et al., 1992; Papadopoulos et al., 1992; Papadopoulos and Guarneri, 1994). Baulieu and colleagues coined the term "neurosteroids" to designate steroids that are synthesized in the CNS and modulate neuronal excitability (Baulieu and Robel, 1990). Regarding this concept, it is noteworthy that a substantial fraction of allopregnanolone may be synthesized de novo in brain (Purdy et al., 1991). Given the possibility that progesterone is reduced to allopregnanolone by glial cells and/or neurons (Barnea et al., 1990; Melcangi et al., 1990), the hypothesis of a progesterone biosynthetic pathway in the brain must be tested.

Once pregnenolone is produced from cholesterol, it

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Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase; $P450_{SCC}$, P450 side-chain cleavage enzyme; 1× SSC, 0.15 *M* NaCl/0.015 *M* sodium citrate (pH 7.6).

may be converted to progesterone. These transformations are catalyzed respectively by specific enzymes including the mitochondrial cytochrome P450 side-chain cleavage enzyme ($P450_{SCC}$), and the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD; see Hanukoglu, 1992, for review). Demonstrating the presence of these enzymes in the CNS has proved difficult, as analytical procedures are hampered by the extremely small amounts expressed in neuronal tissue. For example, RNase protection analysis did not indicate the existence of $P450_{SCC}$ and 3β-HSD mRNAs in adult rat brain (Mellon et al., 1991; Zhao et al., 1991); however, activities of these enzymes were measured in glial cell cultures (Hu et al., 1987; Jung-Testas et al., 1989) and brain tissue extracts (Zhao et al., 1991). More recently, PCR studies suggested the presence of $P450_{SCC}$ in the rat brain (Mellon and Deschepper, 1993) and in situ hybridization with probes against 3β-HSD suggested a localization of this enzyme restricted to neurons within the vestibular and hypoglossal nuclei (Dupont et al., 1994).

In the present study, using sensitive techniques of molecular biology, we attempted to demonstrate the expression in rat brain of mRNAs encoding for $P450_{SCC}$ and 3β-HSD. Analytical PCR was developed to amplify mRNAs from brain structures, as well as from primary glial and neuronal cell cultures. In addition, histoanatomical distributions of $P450_{SCC}$ and 3β-HSD mRNAs were compared by in situ hybridization.

MATERIALS AND METHODS

Primary glial cell cultures

Primary cultures of cortical glial cells were prepared from 4-day-old Sprague–Dawley rat pups. All procedures involving rats were approved by the Georgetown Animal Care and Use Committee. Cerebral hemispheres were isolated under aseptic conditions, and the meninges were carefully removed, to limit contamination by fibroblasts and endothelial cells. The brain tissue was collected in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and penicillin (5 U/ml) + streptomycin (5 μg/ml). Cells were mechanically dissociated by straining through a 17-μm nylon mesh. After plating out the cells, they were placed in a humidified incubator at 37°C under 6% CO_2. The medium was changed 3 days later, and then twice a week. When complete confluence was reached, cells were trypsinized, diluted, and replated at one-fourth their original density. The cells were kept in culture up to 6 weeks.

Oligodendrocyte cultures

Cortical oligodendrocytes were prepared from 2-day-old Sprague–Dawley rat pups according to the procedure of Behar et al. (1988). Oligodendrocyte type 2 astrocyte progenitors were selected with the A_2B_5 monoclonal antibody, which binds to subclasses of surface gangliosides. Cell cultures were enriched in glial A_2B_5-positive precursors by A_2B_5-antibody immunolabeling and differential adhesion selection. DMEM supplemented with 50 μg/ml transferrin, 30 n*M* selenium, 30 n*M* triiodothyronine, 5 μg/ml bovine insulin, and 0.5% FCS was used for growth and differentiation of oligodendrocytes.

Neuronal cultures

Primary cultures of cerebellar granule cells were prepared from 8-day-old rat pups as described previously (Gallo et al., 1982). Cells were plated onto poly-L-lysine–coated 10-cm dishes and cultured in DMEM containing 10% FCS, 2 m*M* glutamine, 50 μg/ml gentamicin, and 25 m*M* KCl at a density of 1.9×10^5 cells/cm^2. Cytosine arabinoside (10 m*M*) was added to all cultures 24 h after seeding to prevent the proliferation of nonneuronal cells. These cultures were characterized as having 90–95% neuronal granule cells.

RNA isolation

Different structures (cortex, cerebellum, and spinal cord) were dissected from adult male Sprague–Dawley rats (200–250 g) and frozen immediately on dry ice for RNA isolation. Frozen tissues were homogenized in RNAzol B (Tel-Test, Inc.) according to the manufacturer's suggestions. The subsequent isolation involved chloroform extraction, isopropanol precipitation, and washing in 70% ethanol. The concentration of total RNA was determined by measuring the optical density at 260 and 280 nm. A similar procedure was used for RNA isolation of cells harvested from cultures.

Oligonucleotides

Primer pairs of 28 and 29 nucleotides were designed in conservative regions from sequences available in the GenBank/EMBL database. Sense primer 5′-ACTCGAGCCAGAAGTATGGCCC(G/C)ATTTA-3′ and antisense primer 5′-AAAGAATTCGAGACACCACCCTCAAATGC-3′ are based on nucleotide sequences of rat and human $P450_{SCC}$ (Chung et al., 1986; Oonk et al., 1989). Restriction sites were introduced to allow the amplified fragments to be subcloned. The sense primer contains an *Xho*I restriction site, and the antisense primer contains an *Eco*RI restriction site. According to the positions of the primers, amplification products of 1,416 bp were expected from the $P450_{SCC}$ mRNA. In additional experiments other PCR primers for $P450_{SCC}$ included the sense primers 5′-AAAGGATCCAGT(G/C)(A/G)CAG(T/C)(T/C)GTGGGGAC-3′ and 5′-ACGCCGTCGACCAGATGTTCCA-3′ and antisense primers 5′-TATCGATGTCTCCTTGATGCTGGCT-3′ and 5′-ATCTGGTCGACGGC(A/G)TC(A/G)ATGA-3′.

Sense primer 5′-TACTCGAGCTCCGCCTGTGATCTGTTTC-3′ and antisense primer 5′-CCCATCGATCTGCTTGGCTTCCTCCCAG-3′ are based on nucleotide sequences of types I and II rat 3β-HSD (Zhao et al., 1990), containing *Xho*I and *Cla*I restriction sites, respectively. The expected 3β-HSD amplification product based on these primers was 1,100 bp. To specifically amplify types III and IV 3β-HSD, the sense primer 5′-TTT(T/C)TGGGCCAGAGGAT(T/C)GTCCA-3′ was used in conjunction with the above antisense primer giving a product of ~1,000 bp.

For a control experiment two 29-nucleotide primers were designed from rat mitochondrial benzodiazepine receptor sequence (Sprengel et al., 1989). Sense primer 5′-AAAGAATTC(T/C)GCCCAG(T/C)CTGGGG(G/T)GCTTC-3′ contains an *Eco*RI site, and antisense primer 5′-TTTAAGCTTCACTCTG(G/T)GAGCCGGGAGCC-3′ contains a *Hin*dIII site, giving an expected amplification product of 487 bp.

Reverse transcription and PCR amplification

The methodology described here was found to be optimal for amplifying rare messages in the brain. When other prod-

ucts were used for reverse transcription and PCR amplification we normally were not able to observe PCR products on ethidium bromide–stained gels. Therefore, the use of each enzyme specified here was crucial for optimal amplification.

A 5-μg aliquot of total RNA from brain structures or cultured cells was reverse-transcribed using 200 units of Superscript RT (GIBCO BRL) for 1 h at 42°C in the presence of oligo(dT) primers following the recommendations of the manufacturer. Residual RNA was then digested 20 min at 37°C with 1 unit of RNase H.

Aliquots of cDNA corresponding to 1 μg of initial total RNA were amplified using a thermal cycler. The amplification mixture contained cDNA, 0.4 μM of each specific primer, 200 μM dNTPs, 2.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 9), 20 mM ammonium sulfate, and 1.5 units of Hot Tub DNA polymerase (Amersham) in a 50-μl volume. The mixture was overlaid with 50 μl of mineral oil and amplified for 35–40 cycles. Amplification included an initial 94°C denaturation step of 5 min, followed by a 5-min 80°C step to allow the addition of the DNA polymerase. Each cycle consisted of a denaturation step (94°C, 45 s), an annealing step (57°C, 30 s), and an elongation step (72°C, 1.5 min), with a final 15-min elongation after the last cycle.

Cloning of PCR products

PCR reaction products were run on a 1% agarose electrophoresis gel containing 1 μg/ml ethidium bromide. The bands of the amplified fragments were recovered and purified with QIAEX DNA-resin (Qiagen) according to the recommendations of the manufacturer. Then the cDNAs were digested with restriction endonucleases (*Eco*RI and *Xho*I or *Cla*I and *Xho*I) overnight at 37°C and the restriction enzymes were inactivated for 15 min at 70°C. Concomitantly, pBluescript KS$^+$ plasmid was digested with the same enzymes, treated with calf intestine alkaline phosphatase for 30 min at 37°C, and "gel-purified" using the conditions described above. PCR products were then ligated to the plasmids at room temperature for at least 2 h in a molar ratio of 1:1. The ligated DNAs were used to transform host *E. coli* strain NM522 for selection and propagation of recombinant plasmids.

Sequencing analysis

DNA sequences were determined by the dideoxy-chain termination method (Sanger et al., 1977) using Sequenase version 2.0 (USB). Samples were analyzed by electrophoresis on 6% polyacrylamide gels containing urea.

cRNA probe preparation

$P450_{SCC}$ and 3β-HSD subcloned in pBluescript KS were linearized with appropriate restriction enzymes for the production of in vitro transcribed ^{35}S-labeled sense and antisense cRNA probes, using the T7 or T3 promoters inherent in pBluescript. The length of the sense and antisense strands generated for $P450_{SCC}$ and 3β-HSD were 150–200 nucleotides. After transcription, residual plasmids were digested for 15 min at 37°C with 1 unit of RNase-free DNase/μg of template. The probes were purified by phenol/chloroform extraction, precipitated in 0.3 M ammonium acetate with 2 volumes of 100% ethanol, and washed with 70% ethanol.

In situ hybridization

In situ hybridization was performed at 55°C with 2×10^6 cpm of either sense or antisense probe, as described by Marlier et al. (1993). The hybridization cocktail was composed of 4× SSC [1× SSC (pH 7) is 0.15 M NaCl, 0.015 M sodium citrate], 120 mM phosphate buffer (pH 7.4), 1× Denhardt's solution, 25 mM β-mercaptoethanol, 20 mM dithiothreitol, 10% dextran sulfate, and 100 μg/ml heat-denatured salmon sperm DNA in 50% formamide. Slides were incubated overnight at 55°C in a humid atmosphere. Then the coverslips were removed in 4× SSC and the slides were placed in 1× SSC, 25 mM β-mercaptoethanol, and 20 mM dithiothreitol for 30 min at room temperature with gentle agitation. Single-stranded RNAs were degraded by incubation for 30 min at 37°C with 40 μg/ml pancreatic RNase A in 10 mM Tris (pH 8), 500 mM NaCl, and 1 mM EDTA. Sections were rinsed successively in 0.5× SSC and 0.1× SSC containing 25 mM β-mercaptoethanol for 30 min at 55°C, dehydrated in graded concentrations of alcohol, and air-dried. Sections were exposed for 5–10 days to Hyperfilm-MP (Amersham) or dipped in NTB-2 emulsion (Kodak) for 3 weeks to reveal the silver grain localization of the hybridized probes. Liquid emulsion–coated sections were stained with neutral red.

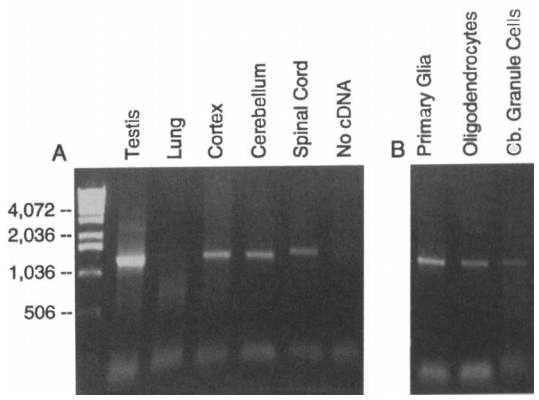

FIG. 1. PCR analysis of $P450_{SCC}$. cDNAs were prepared from 1 μg of RNA subjected to 40 cycles of amplification, except for testis RNA (0.25 μg and 30 cycles), and electrophoresed in ethidium bromide–containing 1% agarose gels. The lane labeled "No cDNA" contained all components of the PCR reaction mixture except that reverse-transcribed cDNA was omitted. Sizes of double-stranded DNA markers are indicated in base pairs. The lower bands in all lanes are due to excess primers.

RESULTS

PCR amplification of rat $P450_{SCC}$ and 3β-HSD cDNA

Figures 1A and 2A show 1% agarose electrophoresis gels of the products amplified by PCR after reverse transcription of 1 μg of total RNA from rat lung, cerebral cortex, cerebellum, and spinal cord, using primers designed from published rat $P450_{SCC}$ and 3β-HSD sequences. Testis RNA (0.25 μg) has been used as a positive control. Negative controls were included, where no reverse-transcribed cDNA was introduced to the amplification reaction. With the designed primers for $P450_{SCC}$ and 3β-HSD single products of the predicted sizes (1.4 and 1.1 kb, respectively) are amplified from testis. In the absence of cDNA no product is observed, indicating that amplification does not result from potentially contaminating sources of these se-

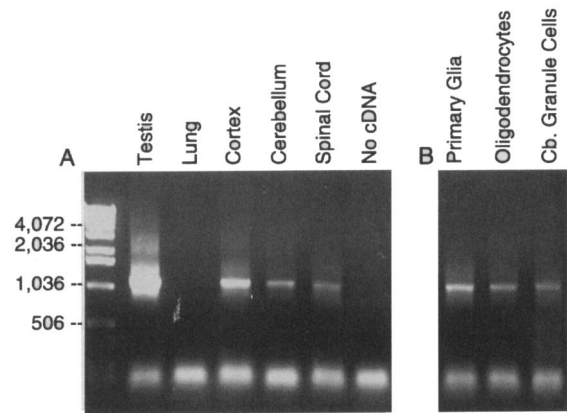

FIG. 2. PCR analysis of 3β-HSD. Reaction conditions and specifications are the same as described for Fig. 1.

quences. From all the brain structures studied, 1.4- and 1.1-kb amplified fragments are detectable at comparable levels after 35–40 cycles. Because both sets of primers span several exons of their respective genes, the expected sizes of each product confirms that amplification was derived from cDNA reverse-transcribed from the mRNA. In other experiments, when reverse transcription was not performed, no amplification product was observed. The intensity of the bands from nervous tissue is very low compared with that obtained from testis. In contrast, even after 40 cycles of amplification, neither $P450_{SCC}$ nor 3β-HSD products were detected from lung, thus serving as an appropriate negative control tissue; however, a 487-bp mitochondrial benzodiazepine receptor fragment (Sprengel et al., 1989) was amplified, verifying the integrity of the RNA from this nonsteroidogenic tissue (data not shown).

Figures 1B and 2B show results obtained in the same conditions from primary glial cell cultures, oligodendrocyte cultures, and cerebellar granule neuronal cultures. After 35–40 cycles, fragments of the predicted sizes are detected at similar levels from all the cultures. As was observed with tissue RNA samples, when reverse transcription was omitted from these cell culture samples no PCR products were evident (data not shown). Furthermore, the PCR products derived from the different cell cultures are clearly not due to contamination by genomic DNA because the respective product sizes are those expected for mature, spliced mRNA sequences lacking the intervening introns found in these genes.

Four isoenzymes of 3β-HSD have been cloned from rat (Zhao et al., 1990, 1991; Simard et al., 1993). To determine which of these subtypes are expressed in the rat brain, different selective PCR primers were designed to amplify either types I and II or types III and IV, concomitantly. In the case where types I and II were selectively amplified from cerebral cortex, a 1-h digestion of the PCR product with either *Nsi*I (which cuts type I 3β-HSD) or *Xba*I (which cuts type II 3β-HSD) gives three bands in each case on the electrophoresis gel (Fig. 3, left). The upper fragment has a size of 1.1 kb, indicative of the uncut PCR product for 3β-HSD, whereas the lower bands reveal both types being cut by the respective restriction enzymes. Sequencing analysis of several subclones derived from this PCR product verifies that both type I and type II are found in the rat brain.

By the same rationale, primers selective for types III and IV also resulted in cDNA amplification from cerebral cortex. In this case no digested products were observed with *Afl*III, an enzyme to specifically cut type III; however, all of the product was cut by *Pst*I, which is specific for type IV (Fig. 3, right). These results suggest that 3β-HSD types I, II, and IV are expressed in the rat brain.

In addition to the types of analyses shown in Fig. 1, the pertinent PCR products for $P450_{SCC}$ were also subcloned into plasmids and sequenced. As the brain has been suspected to express a different $P450_{SCC}$ (Warner et al., 1989), several sets of primers have been used to PCR-amplify its entire coding region. Among multiple PCR products studied, no difference was observed relative to those deposited in the GenBank/EMBL database. This indicates that in the brain,

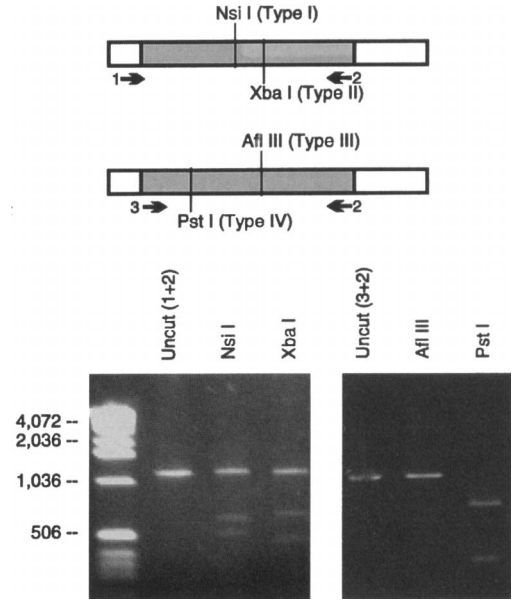

FIG. 3. Identification of 3β-HSD isoenzymes in the rat brain. PCR was performed as in Fig. 2 and the products were digested with the indicated restriction enzymes before electrophoresis in 1.5% agarose gels. The schematic diagram portrays the 3β-HSD mRNA with coding sequence shaded and the locations of PCR primers. Sequences of sense primers 1 and 3 and antisense primer 2 are given in Materials and Methods. Combinations of primers 1 and 2 amplify types I and II, whereas primers 3 and 2 amplify types III and IV. As depicted in the diagram and shown in the gel, restriction enzymes to cut and identify the various cDNA isoenzymes are as follows: type I, *Nsi*I; type II, *Xba*I; type III, *Afl*III; type IV, *Pst*I.

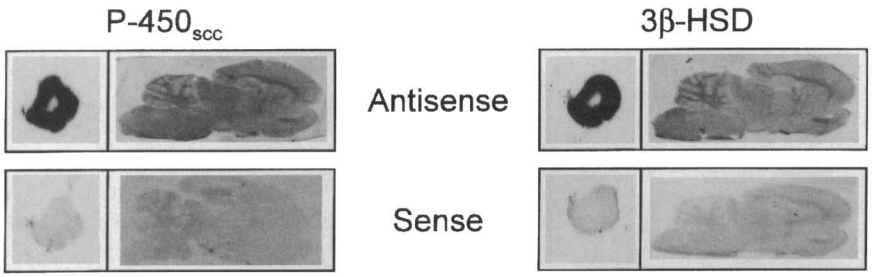

FIG. 4. In situ hybridization of $P450_{SCC}$ and 3β-HSD in the rat brain and adrenal. Sections were prepared and subjected to hybridization as described in Materials and Methods. After hybridization the sections were exposed to Hyperfilm-MP (Amersham) for 8 days. Adrenal and parasagittal brain sections are shown hybridized to different probes as indicated.

the messages for $P450_{SCC}$ and 3β-HSD appear to be identical to those expressed in peripheral endocrine organs. At times various single-base changes were noticed in neural-derived PCR products, but these differences were not consistently observed between separate PCR samples. The inherent low fidelity of the DNA polymerase used in PCR apparently is the cause of these base changes.

Anatomical localization of $P455_{SCC}$ and 3β-HSD mRNAs by in situ hybridization

Sections of adrenal, a classic steroidogenic tissue, have been used to test the specificity of the antisense probes for $P450_{SCC}$ and 3β-HSD (Fig. 4). The adrenal cortex showed strong hybridization signals with both probes, whereas adrenal medulla remained blank. To determine nonspecific hybridization and to establish appropriate washing stringency, the corresponding sense probes were always hybridized in parallel yielding virtually blank images. Parasagittal sections of rat brain demonstrate the widespread distribution of either $P450_{SCC}$ mRNA or 3β-HSD mRNA; however, the signal is faint and an 8-day exposition is necessary (Fig. 4). More intense hybridization is seen in white matter as observed in myelinated structures such as the corpus callosum, internal capsule, the white matter of the cerebellum, and various pathways of the brainstem. Lower levels of hybridization were observed in the gray matter, but this appears to be primarily due to nonspecific hybridization compared with sense probes. Despite the low level of hybridization for both enzyme messages, as was predicted from the PCR analysis, a significant portion of this hybridization in myelinated structures appears specific. From at least five separate in situ hybridization experiments we have found that the antisense probes consistently gave stronger signals in white matter than the background of the sense probes. In all cases sense and antisense probes were nearly equal in length, specific activity, and GC content.

To attempt to better localize the cellular distribution of $P450_{SCC}$ and 3β-HSD mRNAs, the sections were coated with autoradiographic emulsion and exposed for 3 weeks before development. As described above, the regions of myelinated fibers were more highly labeled and the specificity of this hybridization became more evident at the microscopic level. Figure 5 shows a comparison of hybridization intensities in the different layers of the cerebellum. With a control sense probe nearly equal densities of silver grains were found in the granule cell layer and white matter. In contrast, the antisense probes for $P450_{SCC}$ and 3β-HSD showed stronger localization to white matter. The lower signals found in the granule cell layers are comparable with those seen with the sense probe and thus appear to be predominantly nonspecific. Similar comparisons are evident in Fig. 6, showing several myelinated telencephalic structures. The hybridization of the antisense probes were significantly more intense in the corpus callosum, internal capsule, and fimbria compared with the sense probes. It should be noted that the highest nonspecific signal was also found in white matter; however, because the densities of silver grains in gray matter are not significantly different between sense and antisense probes, the relative intensities of signals found in gray and white matter manifest the specific hybridization for both antisense probes in myelinated structures. Because we did not observe significant hybridization above nonspecific levels in gray matter, we could not verify the expression of these mRNAs in neurons by in situ hybridization.

DISCUSSION

Both PCR and in situ hybridization indicate that the rat brain expresses the messages for $P450_{SCC}$ and 3β-HSD mRNAs at very low levels. The reliability of this result is ensured by the coherence of data obtained with two different methods. In addition, the specificity of PCR amplifications is ascertained by negative data from the nonsteroidogenic tissue lung. Quality of lung RNA has been verified by PCR amplification of mitochondrial benzodiazepine receptor mRNA, to guarantee the RNA integrity of this negative control. Moreover, the position of the primers, which allows extension across several exons, demonstrates that amplification was not derived from genomic DNA. Nevertheless, because of the very low mRNA levels, 35–40 PCR amplification cycles are necessary, and in situ–hybridized brain sections required at least 8 days of exposure. Our data do not indicate any appreciable differences in message levels of the major brain structures studied. Quantitative PCR was attempted, but the levels are too low to obtain accurate data. Nevertheless, we estimate that the abundance of both messages is <0.002 fmol/μg, or $<5{,}000$-fold lower than those of rat testis and adrenal. These results are consistent with

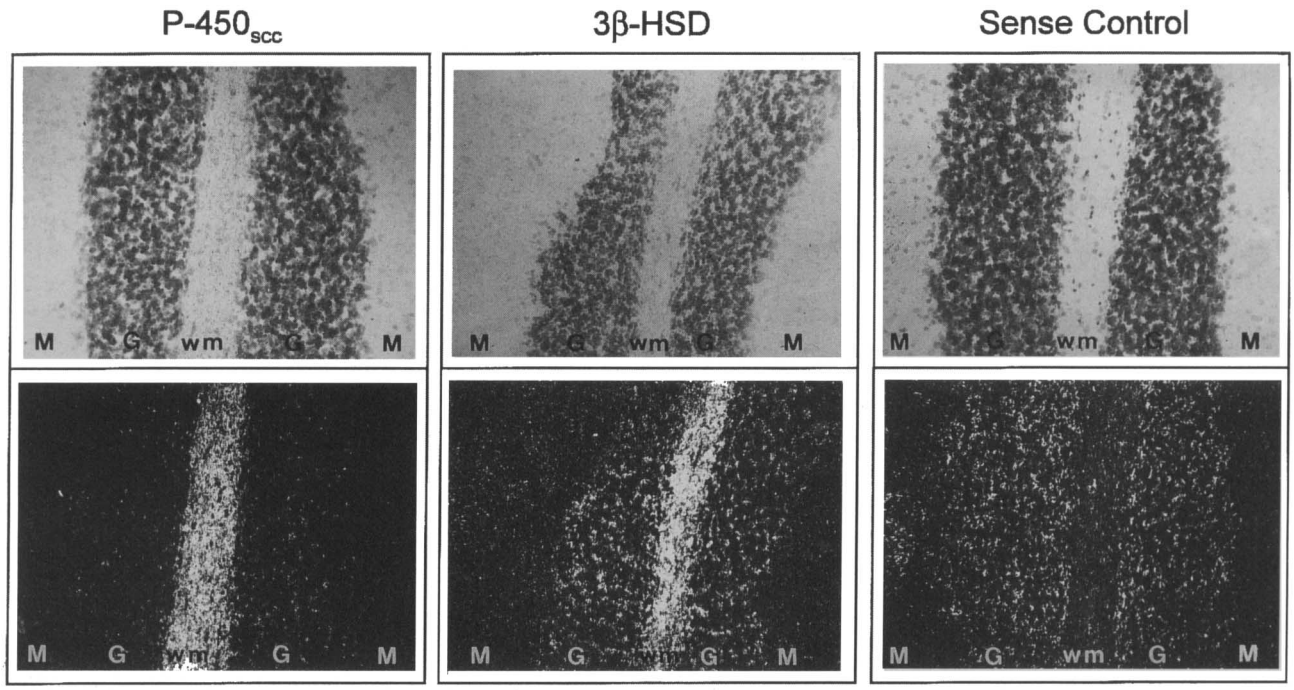

FIG. 5. In situ hybridization within the cerebellum. Brain sections subjected to in situ hybridization were coated with a 1:1 mixture of 300 m*M* ammonium acetate/NTB-2 emulsion (Kodak) and exposed in the dark at 4°C for 3 weeks. After development, the sections were stained with neutral red. The upper panels show bright-field microscopy of the cerebellar cortex above their respective images shown by dark-field microscopy to reveal the silver grains. Sense probe images given here are from 3β-HSD sequence. The different layers of cerebellar cortex are labeled as follows: M, molecular layer; G, granule cell layer; wm, white matter. Note that the exposure time during dark-field microscopy for $P450_{SCC}$ was shortened because of a more intense signal in the white matter.

the demonstration of low levels of $P450_{SCC}$ and 3β-HSD activities in brain (Weidenfeld et al., 1980; Hu et al., 1987; Le Gascogne et al., 1987; Jung-Testas et al., 1989; Iwahashi et al., 1990; Zhao et al., 1991).

The demonstration of brain $P450_{SCC}$ and 3β-HSD mRNA expression was also performed with glial cell cultures from newborn rats. The enzyme mRNAs were hardly detectable with 2-week primary cultures but

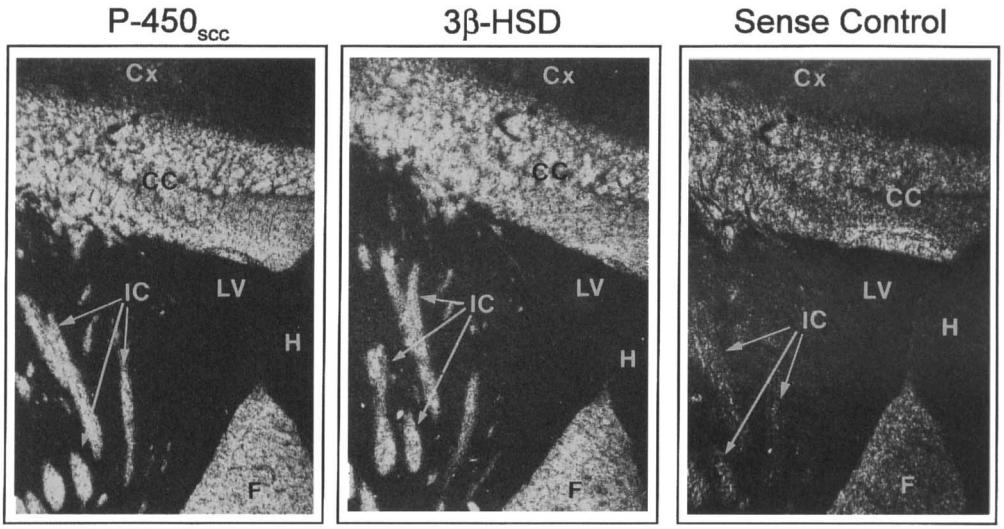

FIG. 6. In situ hybridization of telencephalic structures. Conditions are identical to those described for Fig. 5. Specific structures identified are as follows: CC, corpus callosum; Cx, cerebral cortex; F, fimbria; IC, fiber tracts of the internal capsule coursing through striatum; H, hippocampus; LV, lateral ventricle.

clearly detectable with 3-week primary cultures. These cultures contain primarily type I astrocytes and some oligodendrocytes in a time-dependent ratio. It could be inferred that this result may be due to the oligodendrocyte differentiation process, as suggested in the study of Jung-Testas et al. (1989). It is surprising that in our conditions, preparation of cell cultures enriched in oligodendrocytes does not increase the amount of $P450_{SCC}$ and 3β-HSD mRNAs detected by PCR. This discrepancy may be explained by a translational regulation or the developmental state of the oligodendrocytes. In addition, the expression of these enzymes is also demonstrated in primary cerebellar granule neuron cultures. These neuronal cultures typically contain a 5% subpopulation of astrocytes. Because 35–40 PCR amplification cycles are necessary to detect products from primary glial cell cultures, it is unlikely that this result is from astrocyte origin. In fact, reducing the amount of reverse-transcribed cDNA from granule or glial cultures by three-fold results in the failure to detect PCR products for either enzymes. In conflict with these observations, Kabbadj et al. (1993) have reported that striatal and cerebral cortical neurons did not synthesize progesterone; however, an earlier study on the immunocytochemical localization of $P450_{SCC}$ did note the presence of this enzyme in very select populations of neurons (Le Gascogne et al., 1987), and more recently it has been identified in retinal ganglion cells (Guarneri et al., 1994). Because PCR is more powerful than most biochemical methods, discrepancies can be explained by a difference in the efficacy of the techniques used. It is possible that pregnenolone and progesterone synthesis are restricted to certain neuronal populations and factors leading to the expression of pertinent enzymes may be highly dependent on culturing conditions. We did not attempt PCR analysis from other primary neuronal cell types because glial contamination is much more prominent than that found for cerebellar granule cells.

The presence of four 3β-HSD mRNA species that encode distinct but highly related and functional proteins has been reported in the rat (Zhao et al., 1990, 1991; Simard et al., 1993). Types I, II, and IV were identified in the adult rat brain, as determined using specific PCR primers coupled with discriminative restriction enzymes and sequencing analysis of the amplified brain products. The existence of three 3β-HSD mRNAs offers many possibilities for the regulation of the expression of this enzyme in the brain under various physiological conditions. Type III 3β-HSD shows different substrate and oxidative activities than the other three isoenzymes (Labrie et al., 1992), which may explain its absence in the brain.

In situ hybridization studies were also performed as a sensitive method to determine the localization of $P450_{SCC}$ and 3β-HSD mRNAs within brain sections. Because our antisense probe has >90% sequence identity between 3β-HSD types I, II, and IV, it cannot be discerned whether the various isoenzymes have different expression patterns. Moreover, the in situ hybridization intensity is low as a consequence of the very small amount of $P450_{SCC}$ and 3β-HSD mRNAs in the brain. Nevertheless, our data show a broad distribution of these messages throughout the brain that predominates in white matter. Although nonspecific hybridization often shows a pattern similar to that found for $P450_{SCC}$ and 3β-HSD localization, it does not appear that a high background explains the specific hybridization signals for both enzymes. In multiple experiments the antisense probes consistently showed more intense hybridization compared with the parallel sense probes. Closer analysis at the microscopic level verified a higher specific grain density with myelinated structures, as opposed to gray matter, where the difference between sense and antisense probes was difficult to distinguish. It is noteworthy that this hybridization pattern matches results from immunohistochemical studies of $P450_{SCC}$ (Le Gascogne et al., 1987; Iwahashi et al., 1990). Our results are also in agreement with the demonstration that in the adult rat brain pregnenolone and progesterone appear to be mainly, but not exclusively, synthesized by oligodendrocytes (Hu et al., 1987; Jung-Testas et al., 1989; Kabbadj et al., 1993).

Recently, an in situ hybridization study on 3β-HSD in rat brain was reported (Dupont et al., 1994). The only location where they found specific hybridization was in neurons of the vestibular and hypoglossal nuclei. At first it may appear that our results are in disparity with those of Dupont et al. (1994); however, two important differences regarding the in situ hybridization methods are that they had perfusion-fixed the brain before sectioning and they had used much longer double-stranded cDNA probes where sense control experiments cannot be performed. In some sections we saw a hint of specific hybridization in this region, but the intensity and reproducibility of this signal were difficult to distinguish over nonspecific hybridization. Perhaps prefixation of the tissue better preserves cellular integrity for in situ localization to such a discrete population of neurons. Regarding the second difference in methods used, as white matter typically shows the highest nonspecific signal, they apparently assumed that any signal in these structures was nonspecific. In our experiments the specific hybridization of the 3β-HSD antisense probe in white matter was about two- to threefold higher than that of the sense probes. It is also possible that certain sense cDNA probes resulting from a larger area of 3β-HSD sequence, as produced in the study by Dupont et al. (1994), may incidentally hybridize to another message found within these cranial nerve nuclei. The expression of 3β-HSD in the rat CNS is clearly more widespread than neurons of cranial nerve nuclei VIII and XII, as our PCR data demonstrate that this message is present in the cerebrum, cerebellum, and spinal cord, as well as other structures not shown here.

In contrast with PCR analysis, in situ histological study does not give an indication of $P450_{SCC}$ or 3β-

HSD message expression in the granule cell layer of cerebellum. This disparity may be explained because in situ hybridization is performed on adult rat brain, whereas cerebellar granule cell cultures are developed from newborn rat brain. The cellular state of development may thus be critical for the expression of steroidogenic capability. Alternatively, it is possible that only select neurons may express one or both enzymes at such low levels that they are not readily detected by in situ hybridization.

In conclusion, this study provides molecular biological basis for the proposal that the adult rat brain has the enzymatic capability to convert cholesterol into progesterone, via pregnenolone. Although this study confirms recent work of $P450_{SCC}$ expression in the brain (Mellon and Deschepper, 1993), this is apparently the first demonstration of which 3β-HSD isoenzymes are expressed in the nervous system. Moreover, in situ hybridization suggests that $P450_{SCC}$ and 3β-HSD mRNAs appear to colocalize, as they are mainly expressed in the white matter. It is interesting that it has been reported that the highest concentration of steroid α-reductase is also found in these regions (Celotti et al., 1987). Consequently, it is possible that neuroactive reduced metabolites of progesterone are synthesized de novo in the white matter. Our findings from cell cultures support that the developmental state is critical for the onset of steroid biosynthesis (Akwa et al., 1991) and that, in addition to glial cells, it is possible that certain neuronal cells play a part in the expression of these steroidogenic enzymes during the development of the CNS. The extremely low levels of messages observed highlight the difficulty in studying steroidogenic activity in the CNS. Nevertheless, the expression of the relevant genes has been rigorously examined by PCR in these studies to confirm that the respective mRNAs are in fact detectable by this powerful method.

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