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Neurosteroidogenesis in Astrocytes, Oligodendrocytes, and Neurons of Cerebral Cortex of Rat Brain

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ABSTRACT

The brain is a steroidogenic organ that expresses steroidogenic enzymes and produces neurosteroids. Although considerable information is now available regarding the steroidogenic capacity of the brain, little is known regarding the steroidogenic pathway and relative contributions of astrocytes, oligodendrocytes, and neurons to neurosteroidogenesis. In the present study, we investigated differential gene expression of the key steroidogenic enzymes using RT-PCR and quantitatively evaluated the production of neurosteroids by highly purified astrocytes, oligodendrocytes, and neurons from the cerebral cortex of neonatal rat brains using specific and sensitive RIAs. Astrocytes appear to be the most active steroidogenic cells in the brain. These cells express cytochrome P450 side-chain cleavage (P450scc), 17α-hydroxylase/C17–20-lyase (P450c17), 3β-hydroxysteroid dehydrogenase (3βHSD), 17β-hydroxysteroid dehydrogenase (17βHSD), and cytochrome P450 aromatase (P450arom) and produce pregnenolone (P5), progesterone (P4), dehydroepiandrosterone (DHEA), androstenedione (A4), testosterone (T), estradiol, and estrone. Oligodendrocytes express only P450scc and 3βHSD and produce P5, P4, and A4. These cells do not express P450c17, 17βHSD, or P450arom or produce DHEA, T, or estrogen. Neurons express P450scc, P450c17, 3βHSD, and P450arom and produce P5, DHEA, A4, and estrogen, but do not express 17βHSD or produce T. By comparing the ability of each cell type in the production of neurosteroids, astrocytes are the major producer of P4, DHEA, and androgens, whereas oligodendrocytes are predominantly the producer of P5 and neurons of estrogens. These findings serve to define the neurosteroidogenic pathway, with special emphasis on the dominant role of astrocytes and their interaction with oligodendrocytes and neurons in the genesis of DHEA and active sex steroids. Thus, we propose that neurosteroidogenesis is accomplished by a tripartite contribution of the three cell types in the brain. (*Endocrinology* **140:** 3843–3852, 1999)

EVIDENCE accumulated over the last decade indicated that the brain is capable of *de novo* biosynthesis of neurosteroids independent of gonads, adrenals, and other peripheral steroidogenic organs (for review, see Ref. 1). The first clue of steroidogenesis in brain came from the observation that pregnenolone (P5), dehydroepiandrosterone (DHEA), and their sulfate derivatives accumulate in the brain of castrated and adrenalectomized rats (2). Subsequent *in vitro* studies have confirmed the neurosteroidogenesis activity by the demonstration of gene expression of several steroidogenic enzymes in the brain. Among these enzymes, cytochrome P450 side-chain cleavage (P450scc) messenger RNA (mRNA) was detected in rat brain tissues (3–5), mixed glial cells (6), and enriched oligodendrocytes (7) and astrocytes (8, 9) cell cultures. In a recent study, we presented evidence that astrocytes and neurons of cerebral cortex of rat brain also express 17α-hydroxylase/C17–20-lyase (P450c17) and produce DHEA (10). The previous attempts to demonstrate the presence of P450c17 in astrocytes in culture were unsuccessful (8, 34), as we have shown that the contaminant microglial cells in the cell cultures play a critical role in the biosynthesis of DHEA (10). In the presence of these residual cells, astrocytes produced a negligible concentration of DHEA. Eradication of microglia from astrocyte cell cultures resulted in a dramatic increase in DHEA production (10).

The 3β-hydroxysteroid dehydrogenase (3βHSD) enzyme responsible for the production of progesterone (P4) and androstenedione (A4) was also identified in several regions of adult rat brain (3, 5, 11, 12) and was suggested to be expressed in glial cells (6). Anther key steroidogenic enzyme, 17β-hydroxysteroid dehydrogenase (17βHSD), which catalyzes the conversion of A4 to testosterone (T), was also reported in rat and mouse brain tissue (13–15). In recent studies, we demonstrated cytochrome P450 aromatase (P450arom), mRNA, and activity in cortical and hypothalamic astrocytes of rat brain (10, 16).

Although these studies presented evidence that brain is indeed a steroidogenic organ by its ability to express steroidogenic enzymes and produce neurosteroids *in vitro*, the steroidogenic pathway in astrocytes, oligodendrocytes, and neurons and the relative contribution of these cells to neurosteroidogenesis has not been characterized. The present study was designed to assess the differential gene expression of the key steroidogenic enzymes, including P450scc, P450c17, 3βHSD, 17βHSD, and P450arom, and the production of neurosteroids by cortical astrocytes, oligodendrocytes, and neurons.

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Materials and Methods

Cell culture of cortical glial cells and neurons

Isolation and culture of glial cells (astrocytes and oligodendrocytes). Brains of 1-day-old rats purchased from Charles River Laboratories, Inc. (Wilmington, MA), were used for isolation and preparation of glial and

neuronal cell cultures. Animals were killed on the day of arrival by decapitation. Mixed glial cells were isolated from both cerebral hemispheres by mechanical or mild enzymatic dispersion based on method described by McCarthy and de Vellis (18) with modification by Zwain *et al.* (16, 17). Astrocytes were also isolated from the preoptic/anterior hypothalamic areas. Glial cells were cultured for 10 days in a T75 flask in 10% serum-supplemented Ham's F-12/DMEM at 37 C in a humidified atmosphere. Oligodendrocytes are normally layered on the top of astrocytes. To isolate oligodendrocytes from astrocytes, flasks were shaken at 200 cycles/min for 18 h at 37 C in orbit shaker. Floating cells (oligodendrocytes) were collected, washed, and seeded in culture flasks in 10% serum-supplemented Ham's F-12/DMEM. The attached cells (mainly astrocytes) were washed with fresh medium and shaken again for an additional 15 h to remove any possible contaminant oligodendrocytes. The contaminant microglia cells were eliminated from glial cell cultures by treating cells with L-leucine methyl-ester (LME), which is known as a potent macrophage cytotoxic agent (19, 20). Astrocytes and oligodendrocytes were removed from culture flasks by mild trypsinization and treated in suspension with 10 mM LME for 1 h at room temperature with shaking as previously described (19, 21). As microglial cells are very adhesive cells and will adhere to cell culture substrata within a half-hour, glial cells were successively plated in flasks (three times, 30 min each) with systematic changing of culture flasks as described by Devon (22). This additional purification step was performed to remove any possible remaining contaminant microglia cells. Glial cells were finally plated at various densities in different culture plates. Astrocytes and oligodendrocytes isolated by this method were more than 99% pure as determined by immunocytochemical analysis of glial fibrillary acid protein (GFAP) and galactocerebrosidase (Chemicon, Temecula, CA), which are specific protein markers for astrocytes and oligodendrocytes, respectively. The immunocytochemical analysis of GFAP in astrocytes is shown in Fig. 1. The presence of contaminant endothelial in glial cell cultures was investigated by immunocytochemical analysis of factor VIII protein. No immunoreactive cells were found in the cell cultures. The immunocytochemical analysis of Leu M5 protein, a microglia/macrophage-specific protein marker, was also performed to determine whether microglia cells are eradicated from glial cell cultures. No microglia cells were detected in glial cell cultures after LME treatment and successive plating of glial cells in culture flasks. Before treatment of glial cells with LME, astrocyte and oligodendrocyte cell cultures contained 5% and 3% Leu M5-immunostained cells, respectively. No contaminant neurons were detected in the glial cell cultures as determined by immunocytochemical analysis of neurofilament protein.

The purified astrocytes and oligodendrocytes were extensively washed with serum-free medium (SFM) and cultured for 48 h in SFM with various treatments.

Isolation and culture of neurons. Neuronal culture was performed as previously described by Hertz *et al.* (23) with modification. Briefly, cerebral cortical tissue from 1-day-old rat brains was mechanically dispersed, and cells were cultured in medium containing 20% horse serum for 3 days at 37 C in culture plates precoated with poly-L-lysine substrates. Cell cultures were then exposed for 24 h to cytosine arabinoside (40 μM) to eliminate the nonneuronal cells, including microglia and macroglia cells (astrocytes and oligodendrocytes). To remove any possible remaining contaminant microglia, neurons were successively plated three times

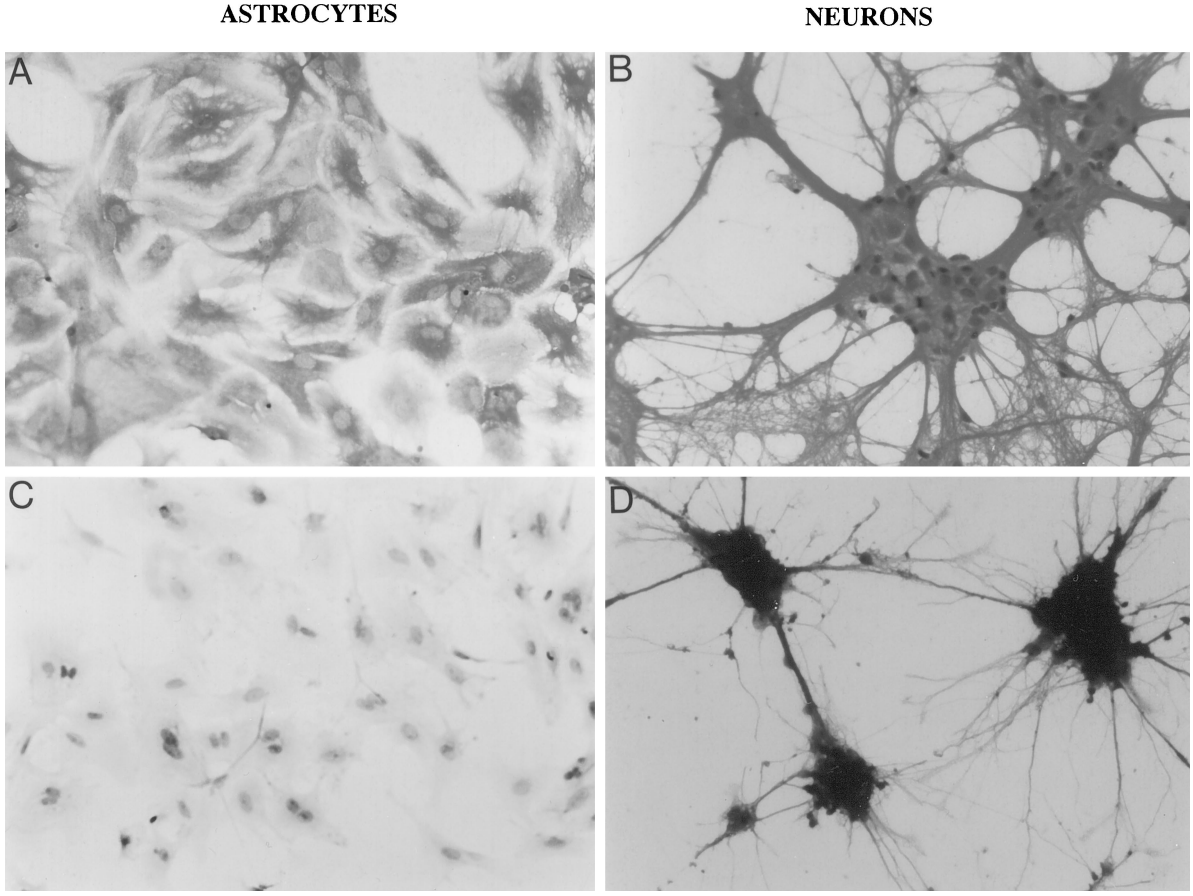

FIG. 1. Immunocytochemical analysis of GFAP in astrocytes (A) and neurofilament protein (NP) in neurons (B). Purified astrocytes and neurons isolated from the cerebral cortex of the neonatal rat brain were cultured on glass coverslips in serum-free medium and immunostained with GFAP or NP, respectively. Positive immunostaining was visualized by Vector red alkaline phosphatase substrate (Vector Laboratories, Inc., Burlingame, CA). Other sets of astrocytes (C) and neurons (D) were immunostained with preimmune serum and used as negative experimental controls. Cells were counterstained with hematoxylin. After 24 h in serum-free medium, neurons were migrating on the coverslips, forming clumps (B and D).

with systematic changing of culture plates as described above. Neurons were then cultured in precoated plates in SFM. After 48 h in culture, neurons were washed and cultured for an additional 48 h in SFM with various treatments. The purity of the neurons was 99%, as judged by immunocytochemical analysis of neurofilament protein (Sigma Chemical Co., St. Louis, MO), a specific protein marker for neurons (Fig. 1). No endothelial, microglial, or glial cells were detected in the neuronal cell cultures, as determined by immunocytochemical analysis.

The purity of isolated neurons, glial cells, astrocytes, and oligodendrocytes was analyzed using cells from three different experiments. Before treatment of cells with steroids, six-well culture plates containing astrocytes, oligodendrocytes, and neurons (one plate of each cell type from each experiment) were randomly selected for assessment of cell purity by immunocytochemical analysis. The isolated cells in the three experiments showed the same percentage of purity (99%).

Preparation of samples

At the conclusion of culture, astrocytes, oligodendrocytes, and neurons were removed by mild trypsinization, and cell viability was determined by trypan blue staining. Media from astrocyte, oligodendrocyte, and neuron cultures were collected, centrifuged, and stored at −20 C until analysis of DHEA, progesterone, androstenedione, testosterone, estrone, and estradiol concentrations using a RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX). The pregnenolone assay was performed using a RIA kit purchased from ICN Biochemicals, Inc. (Costa Mesa, CA). The cross-reactivity of the antisera used in the assay of steroids was determined by the manufacturers of the RIA kits. The minimum detection limit and intra- and interassay variations of P5, DHEA, P4, A4, T, estradiol (E_2), and estrone (E_1) were determined using in-house assays. The minimum detection limits were 25, 24, 90, 70, 60, 2.1, and 3.2 pg/ml; the intraassay variations were 9.13%, 5.86%, 9.25%, 5.70%, 9.60%, 4.85%, and 4.29%; and the interassay variations were 14.16%, 9.09%, 3.79%, 8.70%, 12.86%, 8.66%, and 5.64%, respectively.

Total RNA from cultured cells was isolated using the guanidine phenol-chloroform extraction method and were treated with deoxyribonuclease to remove contaminant DNA. Samples were then stored at −70 C until analysis of the gene expression of steroidogenic enzymes by RT-PCR. The integrity of the RNA was demonstrated by analyzing ribosomal RNAs using ethidium bromide staining.

RT-PCR

RT-PCR analysis of steroidogenic enzymes was performed as previously described by Zwain *et al.* (16). Briefly, 2 μg total RNA were reverse transcribed using Moloney murine leukemia virus RT (Perkin Elmer, Branchburg, NJ) in the presence of oligo(deoxythymidine) primer. One tenth of the RT reaction was used as a template for amplification by PCR using AmpliTaq Gold DNA polymerase (Perkin Elmer). Amplification was performed in a Perkin Elmer thermal cycler with the following cycling parameters: initial activation of the AmpliTaq Gold DNA polymerase at 95 C for 11 min, denaturation at 94 C for 45 sec, annealing at 60 C for 1 min, and extension at 72 C for 2 min. A total of 35 cycles were used, followed by a 10-min final extension at 72 C. The specific sense and antisense oligonucleotide PCR primers for P450scc, P450c17, 3βHSD, 17βHSD, and P450arom are shown in Table 1. PCR products were resolved on 2% agarose gel, stained with ethidium bromide, and visualized under UV light. The identities of PCR products were further confirmed by restriction digestion and Southern blot analyses as previously described (16).

Treatment of astrocytes with oligonucleotides

To determine the specificity of DHEA, testosterone, and estradiol production by astrocytes, the biosynthesis of P450c17, 17βHSD, and P450arom was inhibited by treatment of astrocytes with antisense oligonucleotides to their complementary DNAs (cDNAs) using a transfection kit from Life Technologies, Inc. (Gaithersburg, MD). A 20-mer phosphorothioate antisense and sense oligonucleotides overlapping the initiation codon of the cDNA of each enzyme were designed and HPLC purified. The sense oligonucleotides were used as negative experimental controls. The sequence of the antisense oligonucleotides used to block the biosynthesis of P450arom (24), P450c17, and 17βHSD cDNAs are 5-agcatttccaaaaccatctt-3, 5-cacaagttcccacatggcagc-3, and 5-gcactaccgtggagtccatgtg-3, respectively. Astrocytes were seeded in 12-well plates and cultured for 48 h at 37 C in the presence and absence of antisense (1 and 10 μM) or sense (10 μM) oligonucleotides and with the steroid substrate. At the conclusion of the culture, media were harvested, centrifuged, and stored at −20 C until analysis of steroid concentrations by RIA. The 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide cytotoxicity assay was used to determine the viability of astrocytes treated with the antisense and sense oligonucleotides as previously described by Schlingeniepen and Klinger (25). The 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide assay kit was purchased from Sigma Chemical Co. (St. Louis, MO). Oligonucleotides had no effect on the viability of astrocytes compared with that of controls where cells were cultured alone. The specificity of the antisense oligonucleotide effects on DHEA, testosterone, and estradiol production by astrocytes was investigated by determining its effect on mRNA levels of the steroidogenic enzymes. Change in mRNA levels of the enzymes in treated and untreated astrocytes with sense or antisense oligonucleotides were quantified by semiquantitative RT-PCR, using β-actin as internal standard as previously described (16). Changes in protein levels of the steroidogenic enzymes in the treated astrocytes were not determined because enzymes were present at levels below the detection limit of the Western blot.

Statistical analysis

Student's unpaired *t* test was used when only two groups were compared. For multiple comparisons, data were analyzed by one-way ANOVA with *post-hoc* Bonferroni test using the Statistical Analysis System (StatView software, SAS Institute, Inc., Chicago, IL). Each experiment was repeated two or three times using three replicate cultures for each data point, and the statistical analysis of each set of experiments showed the same results. Results are expressed as the mean ± SD of three replicate cultures. Some data shown no SD because data of the replicate cultures were so similar. $P < 0.05$ was considered statistically significant.

TABLE 1. Oligonucleotides primers used in PCR amplification of steroidogenic enzymes

Primer	Orientation	Sequence	Bases	Length of product (bp)
P450scc (4)	S	caacatcacagagatgctggcagg	965–988	558
	AS	ctcaggcatcaggatgaggttgaa	1523–1500	
P450c17 (4)	S	cccatctattctcttcgcctgggta	227–250	721
	AS	gccccaaagatgtctcccaccgtg	944–921	
3β-HSD (12)	S	ctgcctggtgacaggagcaggagg	186–209	465
	AS	gccagcactgccttctcggccatc	674–651	
17β-HSD	S	gagtggatgtactggtctgt	291–310	747
	AS	ggtttatgtgtgatgcagaccatc	1038–1077	
P450arom (54)	S	ggaggatgacgtgattgacg	1732–1751	334
	AS	ctgtcggggtaacacctt	2065–2048	

S, Sense; AS, antisense. Numbers in *parentheses* are reference numbers.

Results

Gene expression of steroidogenic enzymes

RT-PCR was used to investigate the gene expression of P450scc, P450c17, 3βHSD, 3βHSD, and P450arom in astrocytes, oligodendrocytes, and neurons of neonatal rat brains. RNA was also extracted from ovary and testis and used to determine whether PCR products of steroidogenic enzymes in brain cells are identical to those in gonads. The P450scc was expressed in cortical astrocytes (Fig. 2A, lane 1), oligodendrocytes (Fig. 2A, lane 2), and neurons (Fig. 2A, lane 3). P450c17 was expressed in astrocytes (Fig. 2B, lane 1) and neurons (Fig. 2B, lane 3), but not in oligodendrocytes (Fig. 2B, lane 2). 3βHSD was detected in astrocytes (Fig. 2C, lane 1), oligodendrocytes (Fig. 2C, lane 2), and neurons (Fig. 2C, lane 3). 17βHSD was only expressed in astrocytes (Fig. 2D, lane 1), whereas other cells were negative (Fig. 2D, lanes 2 and 3). P450arom was expressed in astrocytes (Fig. 2E, lane 1) and neurons (Fig. 2E, lane 3). Oligodendrocytes showed no mRNA for P450arom (Fig. 2E, lane 2).

Ovarian (Fig. 2, lanes 4) and testicular (lanes 5) tissue of adult rats abundantly expressed P450scc, P450c17, 3βHSD, 17βHSD, and P450arom (Figs. 2A, 1B, 1C, 1D, and 1E, respectively). The sizes of the RT-PCR products of all steroidogenic enzymes in astrocytes, oligodendrocytes, and neurons were identical to those in ovarian and testicular tissue. The PCR products of steroidogenic enzymes in astrocytes, oligodendrocytes, and neurons as well as in ovarian and testicular tissue were further confirmed by Southern blot analysis using specific internal oligonucleotide probes and by restriction digestion analysis (data not shown). The restriction digestion analysis of RT-PCR products of steroidogenic enzymes in the three cell types of the brain and in ovarian and testicular tissue showed identical DNA fragments. The negative control samples where reverse transcriptase was omitted from the RT-PCR reaction showed no PCR products (data not shown), confirming the specificity of the PCR amplification.

Neurosteroidogenic activity

The neurosteroidogenic activity of P450scc, P450c17, 3βHSD, 17βHSD, and P450arom was evaluated by determining the ability of cortical microglia-free astrocytes, oligodendrocytes, and neurons to produce neurosteroids in the presence of steroid substrates. RIA was used to quantify steroid production by each cell type.

P450scc. Cortical astrocytes, oligodendrocytes, and neurons converted cholesterol into P5 in a dose-dependent manner (Fig. 3, A–C). However, oligodendrocytes were much more active than astrocytes and neurons in the production of P5. At a dose of 10^{-6} M cholesterol, the production of P5 by oligodendrocytes was 3- and 8-fold higher than that by astrocytes and neurons, respectively. Cortical neurons produced low levels of P5.

P450c17. Culture of cortical astrocytes and neurons in the presence of increasing concentrations of P5 resulted in a dose-dependent increase in DHEA production (Fig. 4, A and C, *left panel*). However, astrocytes were more active than neurons in the production of DHEA. At a concentration of 10^{-6} M P5, DHEA production by astrocytes was 10 times higher than that by neurons. Oligodendrocytes were not able to convert P5 into DHEA (Fig. 4B, *left panel*). The production of DHEA is not limited to cerebral cortical astrocytes, as hypothalamic astrocytes were also able to convert P5 into DHEA in a dose-dependent manner (data not shown). The P450c17 activity was further confirmed by the ability of astrocytes and neurons, but not oligodendrocytes, to convert P4 into A4 in a dose-dependent fashion (Fig. 4, A–C, *right panel*).

The specificity of DHEA production by cortical astrocytes was evaluated by treating these cells with increasing concentrations of ketoconazole, a potent inhibitor of cytochrome P450 steroidogenic enzymes, including P450c17. Ketoconazole inhibited the conversion of P5 to DHEA by astrocytes in a dose-dependent manner (Fig. 5A). We also evaluated the specificity of P450c17 activity in astrocytes by blocking its biosynthesis using antisense oligonucleotide specific to P450c17 cDNA. Treatment of astrocytes with the antisense oligonucleotide resulted in a dose-dependent decrease in DHEA production by astrocytes compared with that in the control cells cultured without treatment. The sense oligonucleotide was without effect (Fig. 5B). By semiquantitative RT-PCR using β-actin as an internal standard, the antisense, but not the sense, oligonucleotide has been demonstrated to induce significant inhibition of gene expression of P450c17 compared with the control (data not shown). These data confirm the specificity of the antisense effect.

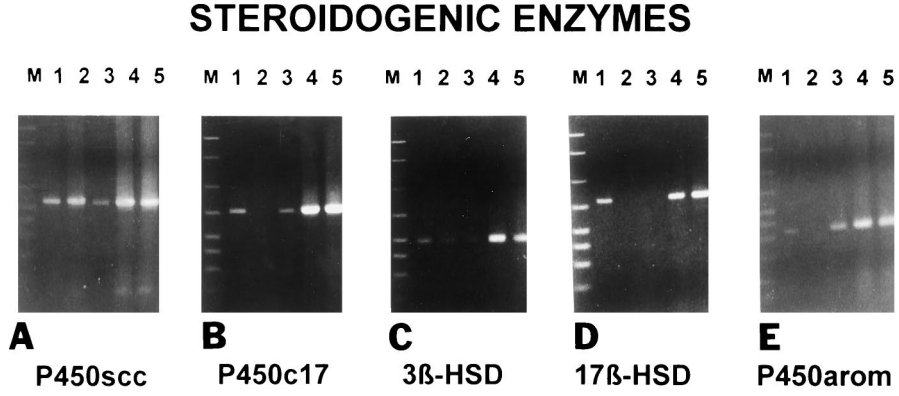

FIG. 2. RT-PCR analysis of steroidogenic enzymes, P450scc, P450c17, 3βHSD, and P450arom in astrocytes (lane 1), oligodendrocytes (lane 2), and neurons (lane 3) from the cerebral cortex of the neonatal rat brain. RNA from ovarian (lane 4) and testicular (lane 5) tissues of adult rats was extracted and used to determine whether PCR products of steroidogenic enzymes in brain cells are identical to those in gonads. M is a DNA size marker.

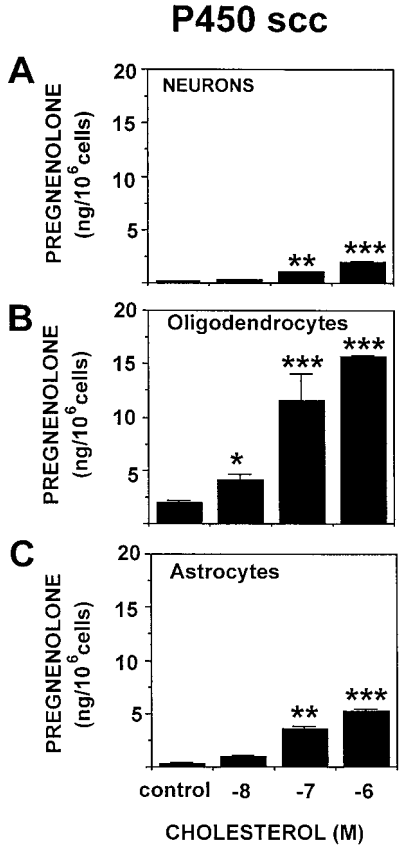

FIG. 3. Cytochrome P450 side-chain cleavage activity in brain as reflected by the conversion of cholesterol to pregnenolone by neurons (A), oligodendrocytes (B), and astrocytes (C) from the cerebral cortex of the neonatal rat brain. *, **, and ***, $P < 0.05$, $P < 0.001$, and $P < 0.0001$ *vs.* control, respectively. Significant differences of at least $P < 0.05$ in pregnenolone production were found among the different groups of neurons, oligodendrocytes, and astrocytes treated with various doses of cholesterol, except no significant difference was found between groups treated with 10^{-7} and 10^{-6} M.

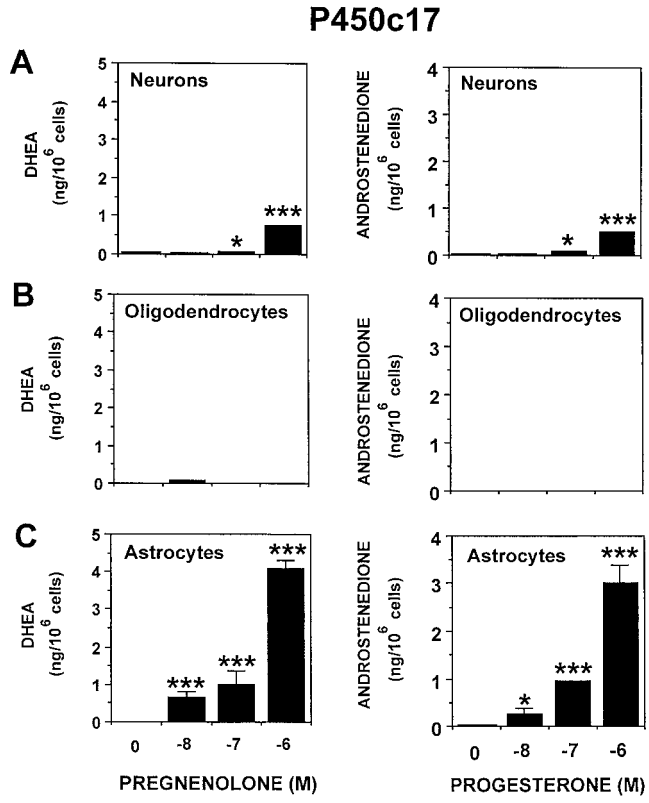

FIG. 4. P450c17 activity in brain, as reflected by the conversion of P5 to DHEA (*left panel*) and P4 to A4 by neurons (A), oligodendrocytes (B), and astrocytes (C) from the cerebral cortex of the neonatal rat brain. * and ***, $P < 0.05$ and $P < 0.0001$ *vs.* control, respectively. Significant differences of at least $P < 0.05$ in DHEA or A4 production were found among the different groups of neurons and astrocytes treated with various doses of P5 or P4, except that between doses 10^{-8} and 10^{-7} M P5 the production of DHEA by astrocytes was not significantly different.

3βHSD. The3βHSD activity in cortical astrocytes, oligodendrocytes, and neurons was also evaluated by determining A4 and P4 production in the presence of DHEA and P5 as substrates, respectively. All three cell types converted DHEA to A4 (Fig. 6, A–C, *left panel*) and P5 to P4 (Fig. 6, A–C, *right panel*) in a dose-dependent manner. Astrocytes were the most active in the conversion of DHEA to A4 and P5 to P4. Production of A4 and P4 by astrocytes was specific, as the addition of trilostane, the 3βHSD inhibitor, to the culture medium resulted in a significant inhibition of the conversion of DHEA to A4 (Fig. 7A) and P5 into P4 (Fig. 7B).

17βHSD. 17βHSD activity was only detected in cortical astrocytes; these cells were able to convert A4 to T in a dose-dependent manner (Fig. 8C). Neither cortical oligodendrocytes (Fig. 8B) nor neurons (Fig. 8A) produced T. The specificity of T production by cortical astrocytes was evaluated by blocking the biosynthesis of 17βHSD by antisense oligonucleotide specific to 17βHSD cDNA. Conversion of A4 to T by astrocytes was inhibited in a dose-dependent manner by treatment of cells with the antisense oligonucleotide compared with that in the untreated cells, whereas the sense oligonucleotide was without effect (Fig. 9). By semiquantitative RT-PCR using β-actin as an internal standard, the antisense, but not the sense, oligonucleotide has been demonstrated to induce significant inhibition of gene expression of 17βHSD compared with the control (data not shown). These data confirm the specificity of the antisense effect.

P450arom. Cortical neurons and astrocytes were shown to aromatize T to E_2 (Fig. 10, A and C, *left panel*) and A4 to E_1 (Fig. 10, A and C, *right panel*) in a dose-dependent manner. However, neurons were more active than astrocytes in the aromatization of androgen to estrogen. Oligodendrocytes do not produce either E_2 (Fig. 10B, *left panel*) or E_1 (Fig. 10B, *right panel*). The antisense oligonucleotide technology was also used to confirm the specificity of estrogen production by astrocytes. Treatment of astrocytes with the antisense oligonucleotide to P450arom cDNA resulted in a dose-dependent inhibition of the aromatization of T to E_2 (Fig. 11) compared with that in the untreated cells. The sense oligonucleotide was without effect. By semiquantitative RT-PCR using β-actin as an internal standard, the antisense, but not the sense, oligonucleotide has been demonstrated to induce significant inhibition of gene expression of P450arom compared with

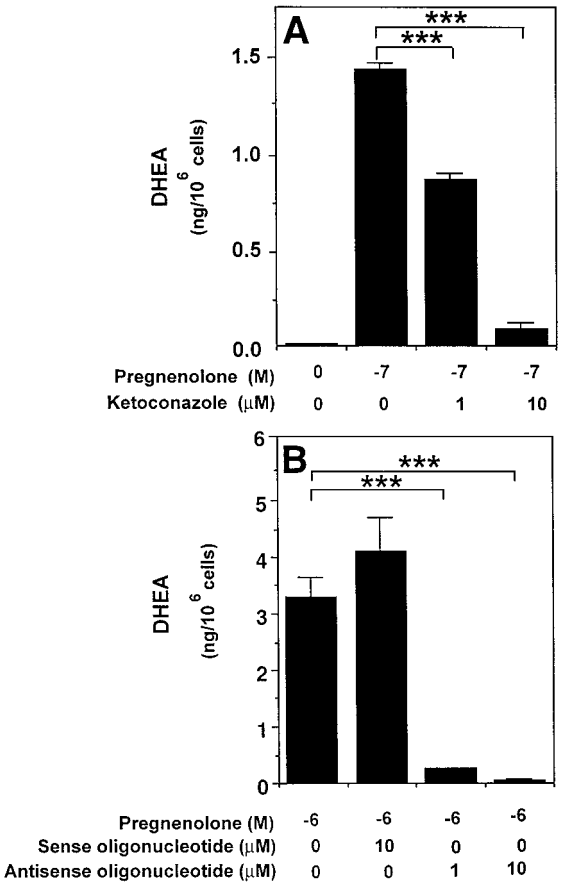

FIG. 5. A, Effect of ketoconazole on the conversion of P5 to DHEA by cortical astrocytes of neonatal rat brains. B, Effect of antisense oligonucleotide specific to P450c17 cDNA on the conversion of P5 to DHEA by cortical astrocytes of neonatal rat brains. ***, $P < 0.0001$. Significant differences of at least $P < 0.001$ in DHEA were found among the different groups of treated astrocytes in A and B.

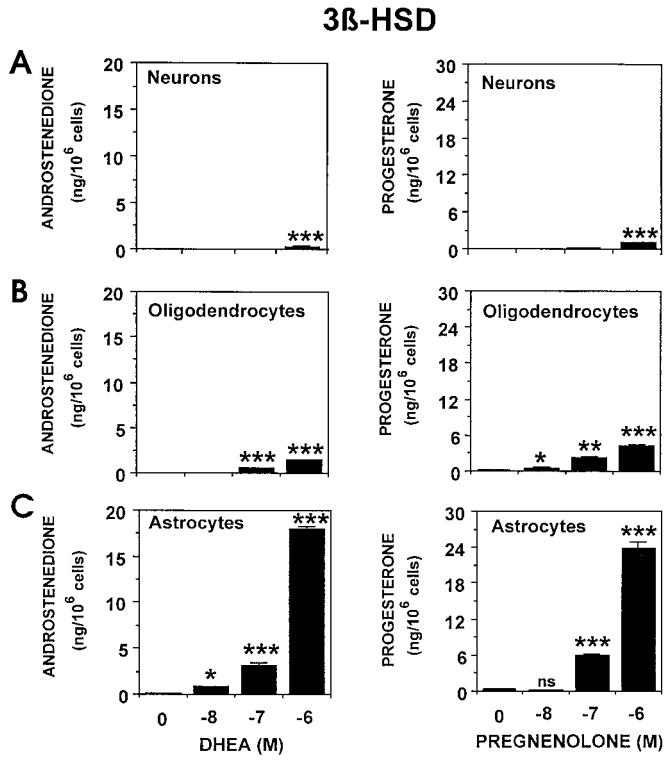

FIG. 6. 3βHSD activity in brain, as reflected by the conversion of DHEA to A4 (*left panel*) and P5 into P4 (*right panel*) by neurons (A), oligodendrocytes (B), and astrocytes (C) from cerebral cortex of neonatal rat brain. *, **, and ***, $P < 0.05$, $P < 0.001$, and $P < 0.0001$ *vs.* control, respectively. Significant differences of at least $P < 0.05$ in androstenedione and progesterone production were found among the different groups of neurons, oligodendrocytes, and astrocytes treated with various doses of DHEA or P5.

that in the control (data not shown). These data confirm the specificity of the antisense effect.

Discussion

The presence of P450scc enzyme and its activity were previously reported throughout the adult rat brain (3–5), mixed glial cells (6), oligodendrocytes (7), and astrocytes (8, 9). In the present study, we have shown the expression of P450scc and the production of P5 by astrocytes, oligodendrocytes, and neurons, providing evidence that cortical neurons are also sites for the expression of P450scc and production of P5 in the brain. However, immunoreactive P450scc was reported in neurons of rat retina (26), peripheral sensory neurons of mouse embryo (27), and the human sciatic nerve (28). Oligodendrocytes are the main source of P5 in the brain, as these cells produce P5 from cholesterol at a level higher than that of astrocytes and neurons, confirming the earliest suggestion that oligodendrocytes are primarily responsible for P450scc activity in the brain (6).

We also showed for the first time that oligodendrocytes express another key steroidogenic enzyme, 3βHSD, and are able to convert P5 to P4 and DHEA to A4. The expression of 3βHSD and the production of P4 and A4 have also been demonstrated in astrocytes and neurons, confirming previous studies reporting the production of P4 from P5 by astrocytes (6) and the presence of 3βHSD and its mRNA in neurons (3, 12).

The production of P4 from P5 by Schwann cells in the peripheral nervous system has been reported (29). Schwann cells share several metabolic characteristics of oligodendrocytes, including myelin formation (1, 29). However, it is not known whether P4 is also involved in myelin formation in the central nervous system and whether it acts directly on neurons or indirectly through oligodendrocytes. Oligodendrocytes have been shown to express myelin proteins, myelin basic protein and 2′,3′-cyclic nucleotide 3′-phosphodiesterase, which are involved in myelin formation (30). As in Schwann cells, P4 was shown to stimulate the expression of myelin proteins in oligodendrocytes (30). Thus, it is possible that as in the peripheral nervous system, P4 may also be involved in myelin formation in the CNS via activation of oligodendrocytes to produce proteins that are necessary for the myelination. Although oligodendrocytes are predominant in the production of P5, the substrate for P4, these cells are less active than astrocytes in the production of P4. This may be due to the low 3βHSD enzymatic activity in oligodendrocytes compared with that in astrocytes. Astrocytes and oligodendrocytes may interact with each other to fulfill their requirements of P4 and P5 that are necessary for their

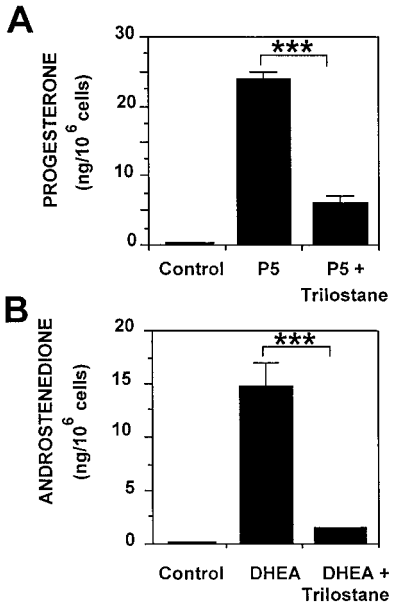

FIG. 7. Effect of trilostane on the conversion of DHEA to A4 (A) and P5 to P4 (B) by astrocytes from the cerebral cortex of the neonatal rat brain. ***, $P < 0.0001$. Significant differences of $P < 0.001$ in A4 and P4 production were found among the different groups of astrocytes cultured alone, with steroid substrate, or with steroid substrate plus trilostane.

functional activities. In this context, astrocytes may provide oligodendrocytes with additional P4, which may be required in the myelination process. Equally, oligodendrocytes may serve as an additional source of P5 for astrocytes to produce P4 and DHEA. Further studies are required to address these issues.

We have also demonstrated that the P450c17 enzyme that catalyzes the conversion of P5 to DHEA is expressed in cortical astrocytes and neurons. However, the presence of this enzyme in brain was a matter of controversy. Compagnone *et al.* (31) detected P450c17 mRNA in rat and mouse embryonic brain tissues and in whole brain and cerebral cortical tissue of young adult rat brains (4, 5, 32). In contrast to these studies, P450c17 protein (33), mRNA (8), and activity (34) were not detected in adult rat brain tissues. Moreover, mixed glial cells and astrocytes from rat and mouse embryo brains in cultures did not express P450c17 mRNA (8) and were unable to metabolize radioactive P5 to DHEA (34). In a recent study, we demonstrated that residual contaminant microglia cells in the cultures dramatically inhibit the production of DHEA by astrocytes *in vitro* (10). By eradication of microglial cells from the cell cultures, astrocytes and neurons become active and produce DHEA (10). Microglial cells were found to be the major contaminants cell type (5%) in the primary astrocyte cell cultures (10, 19). There was no particular effort made by previous studies (8, 34) to eradicate microglial cells from astrocyte cell cultures, and this may explain their failure to detect P450c17, mRNA, and its activity *in vitro*. Although, microglial-free astrocytes and neurons produce DHEA, the gene expression of P450c17 in these cells is low, as it is only detectable by RT-PCR, suggesting that contaminant microglial cells inhibited

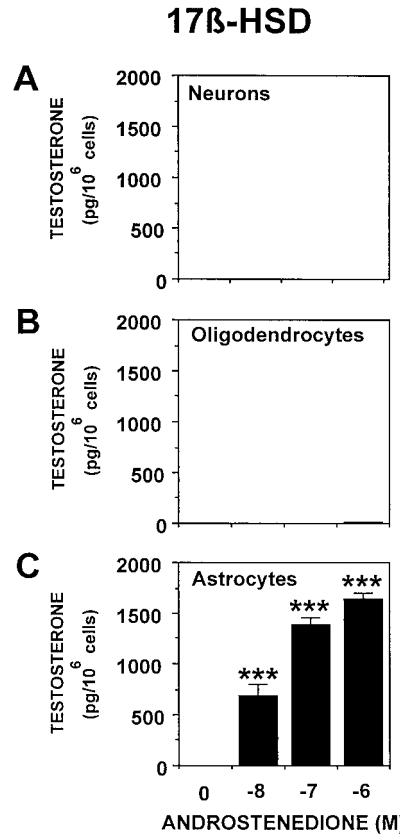

FIG. 8. 17βHSD activity in brain as reflected by the conversion of A4 to T by neurons (A), oligodendrocytes (B), and astrocytes (C) from the cerebral cortex of the neonatal rat brain. ***, $P < 0.0001$ *vs.* control (cells cultured without treatment). Significant differences of at least $P < 0.05$ in T production were found among the different groups of astrocytes treated with various doses of A4.

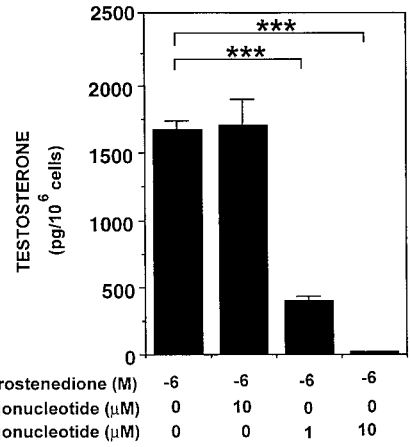

FIG. 9. Effect of antisense oligonucleotide specific to 17βHSD cDNA on the conversion of A4 to T by cortical astrocytes of neonatal rat brains. ***, $P < 0.0001$. Significant differences of at least $P < 0.0001$ in T production were found among the different groups of astrocytes receiving various treatments.

P450c17 mRNA to a level below the detection limit of PCR. However, the production of DHEA by these cells, particularly astrocytes, is relatively high, suggesting a potent enzymatic activity for P450c17 in these cells.

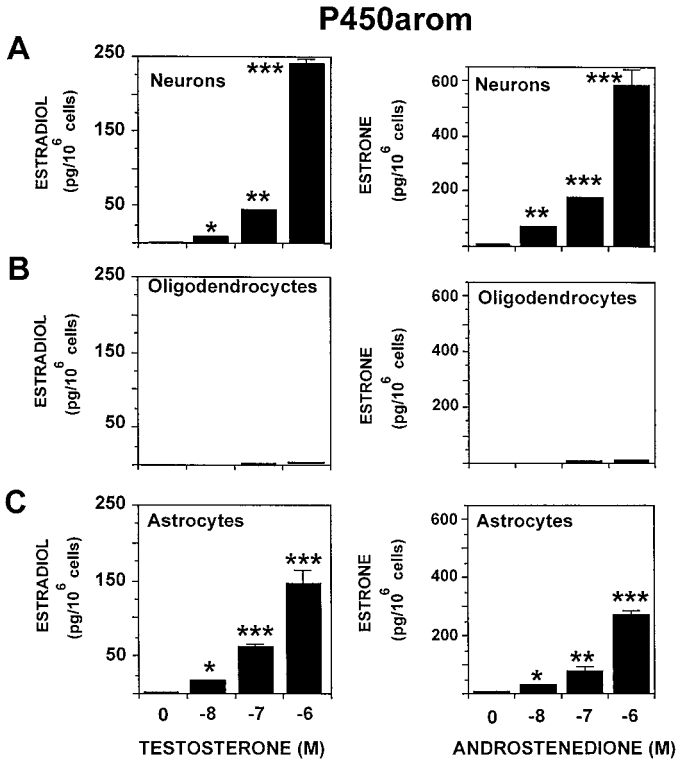

FIG. 10. P450arom activity in brain as reflected by the aromatization of T to E_2 (*left panel*) and A4 to E_1 by neurons (A), oligodendrocytes (B), and astrocytes (C) from the cerebral cortex of the neonatal rat brain. *, **, and ***, $P < 0.05$, $P < 0.001$, and $P < 0.0001$ *vs.* control, respectively. Significant differences of at least $P < 0.05$ in E_2 and E_1 production were found among the different groups of neurons and astrocytes treated with various doses of T or A4.

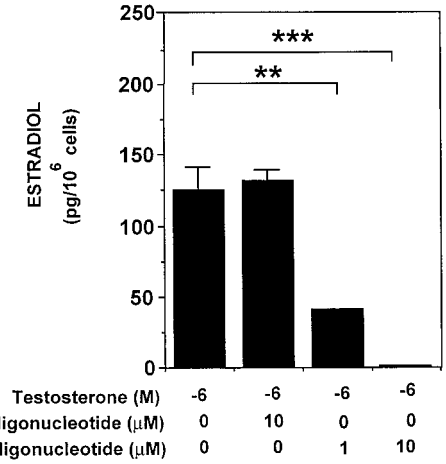

FIG. 11. Effect of antisense oligonucleotide specific to P450arom cDNA on the aromatization of T to E_2 by cortical astrocytes of neonatal rat brains. ** and ***, $P < 0.001$ and $P < 0.000$, respectively. Significant differences of at least $P < 0.0001$ in T production were found among the different groups of astrocytes receiving various treatments.

The inhibition of DHEA production by astrocytes and neurons in culture by microglial cells would not explain the lack of P450c17 and its activity in brain as reported by some *in vivo* studies. In the brain, cellular activity is under the control of a complex interaction of multiple factors, including growth factors, hormones, cytokines, neuropeptides, neurotransmitters, and steroids, that may not exist in the culture dish. The exact mechanism by which microglial cells inhibit DHEA production by astrocytes and neurons in culture is unknown. Microglial cells secret factors such as nitric oxide (NO), tumor necrosis factor-α (TNFα), and interleukin-1β (IL-1β), which are known to be potent inhibitors of gene expression and androgen production in gonads (35–38). The production of NO by microglial cells requires activation of these cells by lipopolysaccharides or β-amyloid (39). However, microglial cells have also been shown to be activated by IL-1 and TNFα to produce NO. IL-1 increases the production and gene expression of TNFα (40), which, in turn, stimulates NO release by microglial cells (41). Further, NO has shown to stimulate TNFα production by microglial cells (41). Based on these studies, cytokines and NO can act mutually to stimulate each other in microglial cells. Thus, it is possible that in response to a signal from microglial cells itself or from astrocytes or neurons in the culture dish, microglial cells become activated and produce high concentrations of cytokines and NO. The possibility that microglial cells may produce other unknown factors that directly inhibit P450c17 gene expression and consequently DHEA production by astrocytes and neurons in culture should also be considered. Further studies are required to address these issues.

The role and significance of DHEA in the brain are not yet known. However, several functional activities have been reported for DHEA in the brain, including modulation of neurotransmitters (42, 43), enhancing memory and learning of adult rats (44, 45), and protection of neurons from damage during some neurodegenerative disorders (46, 47). Additionally, DHEA serves as a substrate for androgen and estrogen biosynthesis by cortical and hypothalamic astrocytes (10). T and E_2 are known to play crucial roles in sexual behavior, neuronal differentiation, and growth (for review, see Ref. 48).

Two other key steroidogenic enzymes, 17βHSD (13–15) and P450arom (49, 50), have been reported in tissues obtained from several regions of rat brain. Data from the present study demonstrated for the first time that astrocytes are the only sites for expression of 17βHSD and production of T from A4 in the brain. Neurons and oligodendrocytes are devoid of 17βHSD and are unable to produce T. We also presented evidence that astrocytes and neurons, but not oligodendrocytes, express P450arom and produce E_2 and E_1 from T and A4, respectively. Neurons appear to be more active than astrocytes in aromatization of androgen to estrogen. As neurons are not able to produce T, it is therefore possible that astrocytes provide T for neurons to produce E_2.

In addition to the steroidogenic enzymes reported in this study, brain has been shown to express several other enzymes, including steroid sulfotransferase, 17α-hydroxysteroid dehydrogenase, 5α-reductase, 3α-hydroxysteroid dehydrogenase, and 7α-hydroxylase, that are required for metabolism of P5, P4, and androgen (1, 51). However, the significance of the biosynthesis of these enzymes and their steroid byproducts in the brain is not known, and further studies are required.

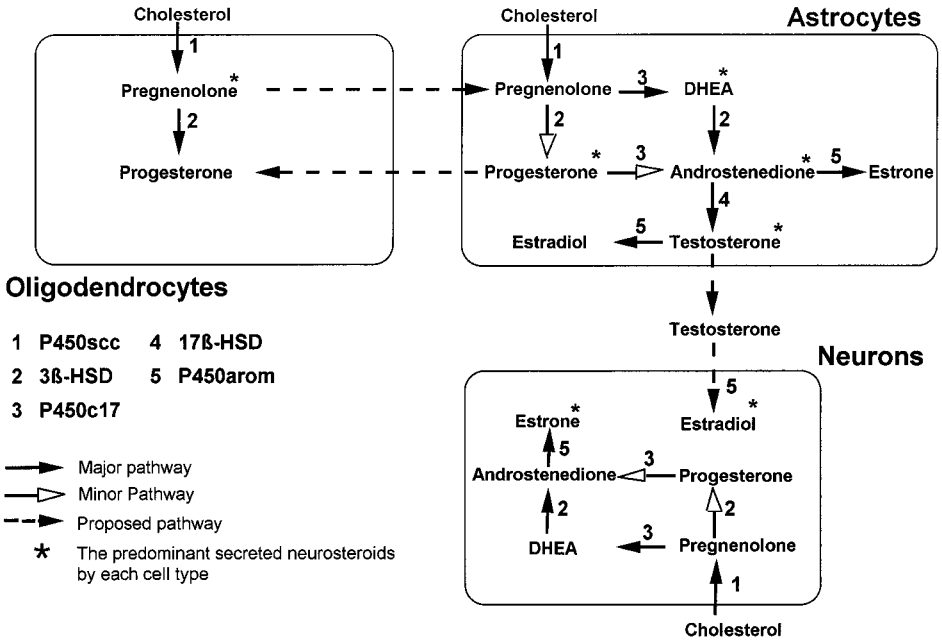

FIG. 12. A schematic view of the neurosteroidogenic pathway in oligodendrocytes, astrocytes, and neurons and potential interaction of these three cell types in neurosteroidogenesis in the rat brain. *Closed arrows*, Major steroidogenic pathway; *open arrow*, minor steroidogenic pathway; *dotted arrows*, proposed steroidogenic pathway. *, The predominantly produced neurosteroids by each cell type.

Summary and conclusion

As illustrated in Fig. 12, astrocytes are the most active steroidogenic cells in the brain, as these cells express P450scc, P450c17, 3βHSD, 17βHSD, and P450arom and produce P5, P4, DHEA, androgens, and estrogens. Oligodendrocytes only express P450scc and 3βHSD and produce P5 and P4. Neurons express the same steroidogenic enzymes and produce the same neurosteroids as astrocytes, with the exception of expression of 17βHSD enzyme and production of T. These data revealed that astrocytes, oligodendrocytes, and neurons have differential capacities in the production of neurosteroids, suggesting that these cells may interact with each other to fulfill their contributions for a full steroidogenic pathway. Oligodendrocytes produce P5 at a level much higher than that in astrocytes, but are less active in the production of P4 that is required for activation of myelin protein formation in oligodendrocytes (30). Astrocytes possess the enzymatic activities to actively convert P5 into P4 and DHEA, which both serve as direct substrates for androgen and estrogen biosynthesis. However, astrocytes produce a small amount of P5 compared with oligodendrocytes. It is therefore possible that oligodendrocytes and astrocytes exchange P5 and P4 that are necessary for maintaining their functional activities (Fig. 12). Although neurons are more active than astrocytes in the aromatization of androgen to estrogen, they lack the enzymatic activity of 17βHSD and are unable to produce T, the direct substrate for E_2 biosynthesis. Astrocytes may therefore play an indirect role in the aromatization process in neurons by providing the substrate T (Fig. 12). We propose that neurosteroidogenesis in the cerebral cortex of the neonatal rat brain is accomplished by a tripartite contribution of the three cell types: astrocytes, oligodendrocytes, and neurons. Further studies are required to validate this theory and determine whether neurosteroidogenesis in the brain is region specific and/or age dependent.

The physiological significance of these locally produced steroids in the brain is not known. The role of steroid in the brain, particularly estrogen, in the regulation of neuronal function has been documented. Steroid receptors, including estrogen, androgen, progestin, and corticosteroid, are widely distributed in brain tissues, confirming a functional system for steroid in the brain. The cellular contributions to the biosynthesis of neurosteroids, as reported in this study, raise several important questions. Does the brain produces neurosteroids to fulfill its need for vital function? Do neurosteroids and circulating steroids have the same effects in the brain? Do neurosteroids and circulating steroids interact with each other in the regulation of neuronal functions? The answers to these interesting questions are beyond the scope of this study, and further investigation is required.

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