

Cytochrome P450 2D Catalyze Steroid 21-Hydroxylation in the Brain

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mRNA of cytochrome P450 21-hydroxylase (P450c21) is expressed in the brain, but little is known about the enzymatic properties of P450c21 in the brain. In the present study, we showed, by using various recombinant cytochrome P450 (CYP)2D enzymes and anti-CYP2D4- or P450c21-specific antibodies, that rat brain microsomal steroid 21-hydroxylation is catalyzed not by P450c21, but by CYP2D isoforms. Rat CYP2D4 and human CYP2D6, which are the predominant CYP2D isoforms in the brain, possess 21-hydroxylation activity for both progesterone and 17 α -hydroxyprogesterone. In rat brain microsomes, these activities were not inhibited by anti-P450c21 antibodies, but they were effectively inhibited by the CYP2D-specific chemical inhibitor quinidine and by anti-CYP2D4 antibodies. mRNA and protein of CYP2D4 were

expressed throughout the brain, especially in cerebellum, striatum, pons, and medulla oblongata, whereas the mRNA and protein levels of P450c21 were extremely low or undetectable. These results support the idea that CYP2D4, not P450c21, works as steroid 21-hydroxylase in the brain. Allopregnanolone, a representative γ -aminobutyric acid receptor modulator, was also hydroxylated at the C-21 position by recombinant CYP2D4 and CYP2D6. Rat brain microsomal allopregnanolone 21-hydroxylation was inhibited by fluoxetine with an IC₅₀ value of 2 μ M, suggesting the possibility that the brain CYP2D isoforms regulate levels of neurosteroids such as allopregnanolone, and that this regulation is modified by central nervous system-active drugs such as fluoxetine. (*Endocrinology* 145: 699–705, 2004)

STEROID 21-HYDROXYLATION IS an obligatory conversion for the biosynthesis of the main adrenal steroids, aldosterone, cortisol, and corticosterone. In the adrenal gland, cytochrome P450 21-hydroxylase (P450c21) converts progesterone and 17 α -hydroxyprogesterone to 11-deoxycorticosterone and 11-deoxycortisol, respectively. However, there is no published evidence concerning *de novo* synthesis of adrenal steroids by P450c21 in the brain, and although the brain possesses 21-hydroxylase activity (1), it expresses only a very small amount of P450c21 mRNA (2, 3). These findings, and the residual 21-hydroxylase activity found in human patients with congenital adrenal hyperplasia due to a defective P450c21 gene (4, 5), suggest the existence in the brain of another 21-hydroxylase different from the adrenal P450c21.

Cytochrome P450 (CYP)2D4 and CYP2D6 are CYP2D isoforms predominantly expressed in the brains of rats and humans, respectively (6–10). Recent studies have revealed that CYP2D isoforms contribute not only to the metabolism of a large number of clinically relevant drugs in the liver, but also to the biosynthesis of endogenous substances such as dopamine (11). In addition, we showed previously that CYP2D4 and CYP2D6 generate 11-deoxycorticosterone

through 21-hydroxylation of progesterone, and that this activity in rat brain microsomes could be effectively inhibited by anti-CYP2D4 antibodies (12). Thus, we hypothesized that the CYP2Ds, not P450c21, are responsible for steroid 21-hydroxylation in the brain. The physiological functions of the brain CYP2D isoforms have not so far been fully elucidated.

In the present study, we investigated whether CYP2D isoforms catalyze steroid 21-hydroxylation in the brain by using various recombinant CYP2D enzymes. In addition to progesterone and 17 α -hydroxyprogesterone, allopregnanolone was recognized to be a substrate for brain CYP2D, based on the effects of specific antibodies against CYP2D4 or P450c21. We also examined regional variations of CYP2D4 and P450c21 in brain at the mRNA and protein levels by using RT-PCR and Western blot analysis.

Materials and Methods

Chemicals and animals

Progesterone, 11-deoxycorticosterone, 17 α -hydroxyprogesterone, 11-deoxycortisol, allopregnanolone, 3 α ,5 α -tetrahydrodeoxycorticosterone, 5 α -dihydroprogesterone, 5 α -dihydrodeoxycorticosterone, quinidine, and fluoxetine were purchased from Sigma-Aldrich (St. Louis, MO). Bufuralol was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) was obtained from the Oriental Yeast Co., Ltd. (Tokyo, Japan). Other reagents and organic solvents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Male Sprague Dawley rats, obtained from Nippon Clea (Kyoto, Japan), were maintained in a 12-h light, 12-h dark cycle (lights on at 0600 h) with free access to water and pelleted food.

Abbreviations: CYP, Cytochrome P450; GABA, γ -aminobutyric acid; LC-MS/MS, liquid chromatography/tandem mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); P450c21, cytochrome P450 21-hydroxylase; RT, reverse transcriptase; V_{max}, maximum velocity.

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Recombinant CYP2D-expressing microsomes and antibody

Rat CYP2D1 and CYP2D3 cDNAs were isolated from a rat liver cDNA library. Rat CYP2D2 cDNA was amplified from a rat liver cDNA library by PCR. Rat CYP2D4 cDNA was amplified from rat brain total RNA by RT-PCR. Human CYP2D6 cDNA was amplified from a human liver cDNA library by PCR. CYP2D1, CYP2D2, CYP2D3, CYP2D4, and CYP2D6 enzymes were expressed in *Saccharomyces cerevisiae*, as reported previously (13, 14), and the microsomal fraction was prepared from the yeast cells and used as recombinant enzymes. The contents of recombinant CYP2D1, CYP2D2, CYP2D3, CYP2D4, and CYP2D6 were 95.5, 49.0, 22.8, 90.7, and 56.4 nmol/mg, respectively.

Anti-CYP2D4 antibodies were raised in a female Japanese White rabbit using recombinant CYP2D4 as the immunogen. Anti-CYP2D4 antibodies recognized rat CYP2D4 and inhibited CYP2D4 catalytic activity. Anti-P450c21 antibodies, elicited in a male white rabbit using purified bovine adrenocortical microsomes as the immunogen (15), recognized and cross-reacted to rat P450c21.

Preparation of rat brain microsomes

Adult male rats were killed by decapitation, and their brains, livers, and adrenal glands were immediately removed by dissection. Brains were divided into nine regions: cerebrum, cerebellum, midbrain, medulla oblongata, hippocampus, thalamus, hypothalamus, pons, and striatum. Rat tissue microsomes from each brain region were prepared as described previously, with some minor modifications (16). All animal treatments were performed according to standard methods of humane animal care. The protocol for this study was approved by the Committee on Animal Care and Use of Osaka City University Medical School.

Microsomal steroid 21-hydroxylation

Progesterone, 17 α -hydroxyprogesterone, 5 α -dihydroprogesterone, or allopregnanolone (12.5 nmol) was incubated at 37 C with rat brain microsomes (0.5 mg protein) or recombinant CYP2D isoforms (90 pmol) in a final volume of 0.5 ml 0.1 M potassium phosphate buffer (pH 7.4). To study chemical inhibition, various amounts of quinidine or fluoxetine were added at a final concentration of organic solvent of less than 0.5%. For the immunoinhibition study, anti-CYP2D4 and anti-P450c21 antibodies were used. Rat brain microsomes were preincubated with various amounts of anti-CYP2D4 or anti-P450c21 antibodies at room temperature for 15 min. After the preincubation, substrate and 0.1 M potassium phosphate buffer (pH 7.4) were added to the mixture on ice. The reaction was initiated by adding NADPH (0.5 μ mol) and terminated with the addition of 2 ml ethyl acetate. After extraction with ethyl acetate, the organic phase was collected and evaporated under vacuum on a rotary evaporator. The resulting residue was dissolved in methanol and injected onto a HPLC apparatus with an ODS-80Ts column (2.0 \times 150 mm; Tosoh Corporation, Tokyo, Japan). Bufuralol 1'2'-ethenylation activity (a probe specific to rat CYP2D4) was measured as described previously (17).

Quantification of metabolites were performed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) methods as described previously, with minor modifications (12). To quantify metabolites, selected-reaction monitoring mode was applied for 11-deoxycorticosterone (m/z 331.2–295.2), 11-deoxycortisol (m/z 347.2–311.2), 5 α -dihydrodeoxycorticosterone (m/z 315.2–278.2), and 3 α ,5 α -tetradeoxycorticosterone (m/z 317.2–281.2). The LCQ instrument was interfaced to a computer workstation running Finnigan Mat LCQ Navigator software. Microsomal activity was quantified from the peak area, using linear calibration plots obtained by LC-MS/MS.

Expression of mRNA and protein in the brain

Total RNA was extracted from frozen tissue homogenates using total RNA isolation reagent (Nippon Gene, Toyama, Japan). After deoxyribonuclease treatment, total RNA was reverse-transcribed using an RNA PCR kit (Takara Shuzo Co., Ltd., Kyoto, Japan) according to the manufacturer's instructions. For conversion of total RNA to cDNA, a reaction mixture (20 μ l) was prepared containing 5 U reverse transcriptase (RT) avian myeloblastosis virus (Takara Shuzo), 1 \times RT-PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 1.5 mM MgCl₂, 1 mM deoxynucleotide

triphosphates, 2.5 μ M random 9-mer primers, 20 U ribonuclease inhibitor, and 2 μ g total RNA. The reaction was carried out at 55 C for 60 min. RT was terminated by heating the reaction mixture to 99 C for 5 min, followed by rapid chilling on ice. RT reaction mixtures, including cDNA products, were stored at –20 C until used. A single cDNA produced from total RNA was amplified by PCR with primers for CYP2D4, and P450c21. Specific oligonucleotide primer pairs for these isoforms were synthesized. Sense and antisense primer sequences for CYP2D4 were 5'-GACCAGTCGGGCTTTGGACCAC-3' and 5'-CGAAGGCCTTCTTCCAGAG-3' (nucleotide positions 3094–3115 and 4179–4198 in the rat CYP2D4 gene sequence), respectively (18). Those for P450c21 were 5'-AGGAGCTGAAGAGGCACAAG-3' and 5'-GAGGTAGCTGCATTGCGTTC-3' (nucleotide positions 719–738 and 1005–1024 in the rat CYP21 gene sequence), respectively (19). The expected product sizes from CYP2D4 and P450c21 were 420 and 306 bp, respectively. For PCR amplification of cDNA, the PCR was allowed to proceed for 35 cycles in 50- μ l aliquots of reaction mixture containing 1 \times polymerase reaction buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 1.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates, 1 U Gold Taq DNA polymerase (Applied Biosystems, Foster City, CA), 0.1 μ g cDNA and 200 nM concentration of the specific primers. Aliquots (10 μ l) of amplified cDNA products were separated by electrophoresis using 2.0% agarose gels. Gels were stained with ethidium bromide and visualized under UV light. Images of the gels were recorded with a Color Image Freezer (Atto Corp., Tokyo, Japan).

The microsomal fractions were denatured for 5 min at 90 C and subjected to electrophoresis using 10% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) for 90 min. Blotted membranes were washed three times with PBS containing 0.05% Tween 20 and blocked for 15 min. Blots were then probed with antibodies against CYP2D4 (1:500) or P450c21 (1:500) for 60 min at room temperature. After primary antibody treatment, the blots were washed and treated with a Vectastain avidin/biotin complex kit (Vector, Burlingame, CA) according to the manufacturer's protocol. The protein bands were detected with 4-chloro-1-naphthol (Bio-Rad). For quantitative analysis, images of the bands were captured with a scanner, and densitometric analysis was performed using NIH Image 1.61 software.

Results

Steroid 21-hydroxylation by recombinant CYP2D isoforms

One isoform (CYP2D6) in humans and four major isoforms (CYP2D1, CYP2D2, CYP2D3, and CYP2D4) in rats have been identified (18, 20). We investigated whether these rat and human CYP2D isoforms catalyze steroid 21-hydroxylation. The microsomal fraction containing CYP2D1, CYP2D2, CYP2D3, CYP2D4, or CYP2D6 prepared from yeast cells, was used as recombinant enzyme (13, 14). Each recombinant enzyme was incubated with progesterone or 17 α -hydroxyprogesterone, and the 21-hydroxylated product, 11-deoxycorticosterone or 11-deoxycortisol, respectively, was identified by LC-MS/MS and quantified by comparison with an authentic sample. As shown in Fig. 1, rat CYP2D1, CYP2D2, and CYP2D3 had no 21-hydroxylation activity toward progesterone or 17 α -hydroxyprogesterone. In contrast, rat CYP2D4 possessed 21-hydroxylation activity toward both progesterone and 17 α -hydroxyprogesterone, amounting to 1.06 \pm 0.17 and 0.085 \pm 0.005 pmol/min \cdot pmol P450, respectively. In humans, the progesterone and 17 α -hydroxyprogesterone 21-hydroxylation activities of CYP2D6 were 0.18 \pm 0.09 and 0.062 \pm 0.005 pmol/min \cdot pmol P450, respectively. These results indicate that rat CYP2D4 and human CYP2D6, the predominant CYP2D isoforms in the brain, can catalyze 21-hydroxylation of steroids.

The kinetic parameters of recombinant CYP2D4-mediated 21-hydroxylation of progesterone and 17 α -hydroxyproges-

terone were calculated (Table 1). The Michaelis-Menten constant (K_m) values for progesterone and 17α -hydroxyprogesterone were 15.4 ± 2.9 and $17.6 \pm 5.6 \mu\text{M}$, respectively, which were higher than those obtained from P450c21 recombinant enzyme expressed in the yeast (*i.e.* 0.33 and $0.23 \mu\text{M}$, respectively) (21). The maximum velocity (V_{max}) values were 1.58 ± 0.10 and $0.14 \pm 0.02 \text{ pmol/min}\cdot\text{pmol P450}$, respectively. The levels of steroid hormones in the blood circulation are very low (22); however, the levels in the steroidogenic tissues are thought to be relatively high. In fact, the K_m value of the steroidogenic CYP isoform CYP7 for steroid hormone was reported to be about $13 \mu\text{M}$ (23). The K_m values of CYP2D4 for these 21-hydroxylations are probably sufficient for the *in vivo* reaction to be significant.

Steroid 21-hydroxylation by rat brain microsomes

CYP2D4 is known to be expressed in rat brain (6, 8, 9). We therefore examined the contribution of CYP2D4 to rat brain microsomal steroid 21-hydroxylation. Rat brain microsomes catalyzed 21-hydroxylation of progesterone and 17α -hydroxyprogesterone with activities of 0.051 ± 0.035 and $0.024 \pm 0.010 \text{ pmol/min}\cdot\text{mg protein}$, respectively, at the substrate concentration of $25 \mu\text{M}$. To investigate which enzyme (*i.e.* CYP2D4 or P450c21) is the main mediator of rat brain microsomal 21-hydroxylation, the CYP2D-specific inhibitor quinidine, or anti-CYP2D4 antibodies, or anti-P450c21 antibodies were added to the reaction mixture of rat brain microsomes. Quinidine and anti-CYP2D antibodies did not in-

hibit rat adrenal progesterone or 17α -hydroxyprogesterone 21-hydroxylation, which is catalyzed by adrenal P450c21 (data not shown). Furthermore, anti-P450c21 antibodies did not inhibit rat brain microsomal bufuralol 1'2'-ethnylation, which is a specific reaction for CYP2D4 (17), but inhibited rat adrenal microsomal P450c21 catalytic activities (data not shown). As shown in Fig. 2, A and B, the rat brain microsomal 21-hydroxylation of both progesterone and 17α -hydroxyprogesterone was almost completely inhibited by quinidine in a dose-dependent manner. These activities were not inhibited by anti-P450c21 antibodies, but were effectively inhibited by anti-CYP2D antibodies (Fig. 2, C and D). These results support the view that rat brain microsomal steroid 21-hydroxylation is catalyzed not by P450c21, but by CYP2D4.

Regional distribution of CYP2D4 and P450c21 in rat brain

To examine the expression of CYP2D4 and P450c21 mRNAs in rat brain, total RNA extracted from each brain region was subjected to RT-PCR. RNA extracts of the liver and adrenal gland served as positive controls of CYP2D4 and P450c21, respectively. The initial RNA amount provided for RT-PCR was adjusted on the basis of spectrophotometric

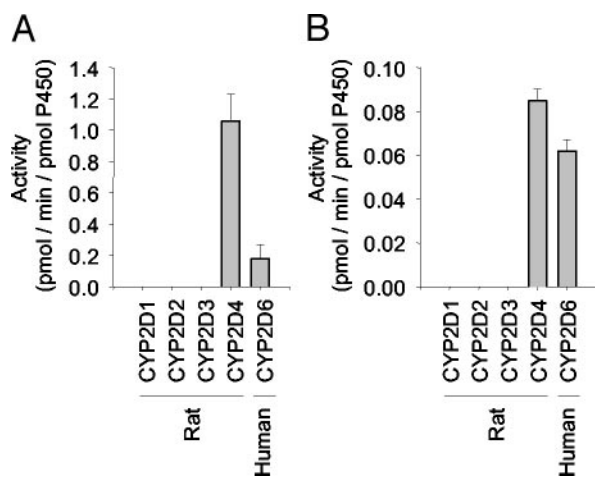


FIG. 1. Steroid 21-hydroxylation by recombinant CYP2D-expressing microsomes. Recombinant microsomes expressing various CYP2D isoforms (CYP2D1, -2D2, -2D3, -2D4, and -2D6) were incubated with 12.5 nmol progesterone (A) or 17α -hydroxyprogesterone (B) in a final volume of 0.5 ml 0.1 M potassium phosphate buffer (pH 7.4). Data are expressed as the mean \pm SD ($n = 3$).

TABLE 1. Kinetic parameters of steroid 21-hydroxylation by CYP2D4

Substrates	K_m	V_{max}	V_{max}/K_m
Progesterone	15.4 ± 2.9	1.58 ± 0.10	0.106 ± 0.024
17α -Hydroxyprogesterone	17.6 ± 5.6	0.14 ± 0.02	0.008 ± 0.001

K_m values and V_{max} values are expressed as micromolar concentrations and picomoles per minute per picomoles of P450, respectively. Data are expressed as the mean \pm SD ($n = 3$).

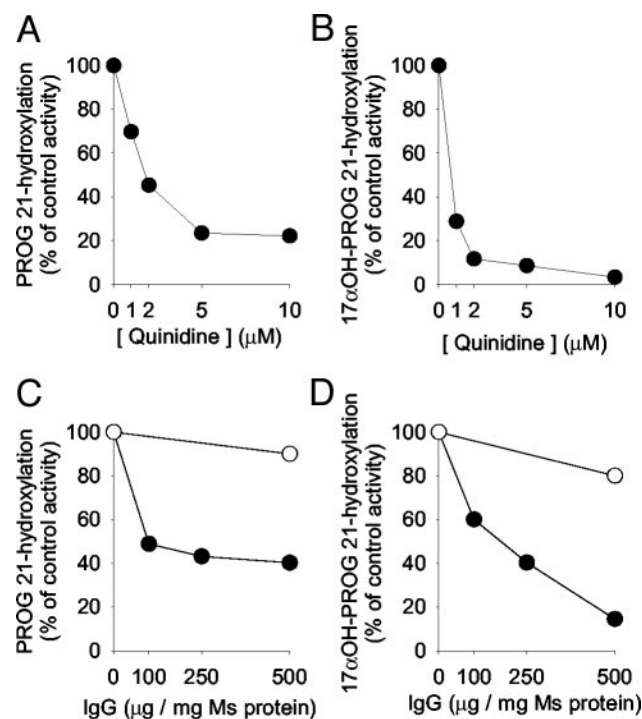


FIG. 2. Chemical inhibition and immunoinhibition of rat brain microsomal steroid 21-hydroxylation. Rat brain microsomes (0.5 mg) were incubated with 12.5 nmol progesterone (A) or 17α -hydroxyprogesterone (B) in the presence of various concentrations of the CYP2D-specific inhibitor quinidine in a final volume of 0.5 ml 0.1 M potassium phosphate buffer (pH 7.4). Rat brain microsomes (0.5 mg) were preincubated with various amounts of anti-CYP2D antibodies (filled circle) or anti-P450c21 antibodies (open circle) at room temperature for 15 min. After preincubation, 12.5 nmol progesterone (C) or 17α -hydroxyprogesterone (D), and 0.1 M potassium phosphate buffer (pH 7.4) were added to the mixture on ice. The reaction was initiated by adding NADPH ($0.5 \mu\text{mol}$). Progesterone (A and C) and 17α -hydroxyprogesterone (B and D) 21-hydroxylation activities were expressed as percentage of the control.

measurement, and RT-PCR for glyceraldehyde-3-phosphate dehydrogenase was performed as an internal standard. In gel electrophoresis of the RT-PCR product for the CYP2D4 gene (Fig. 3, A and B), a single band corresponding in size to CYP2D4 mRNA, but not the genomic size, was detected in all regions assayed here, *i.e.* cerebrum, cerebellum, midbrain, medulla oblongata, hippocampus, thalamus, hypothalamus, pons, and striatum. The density of the band in each brain region was lower than that in the liver, but all regions except for cerebellum gave bands of similar density. The expression of P450c21 mRNA was also examined using the same RNA samples (Fig. 3, A and C). P450c21 mRNA expression was not detected in the cerebrum, medulla oblongata, or hypothalamus. In other brain regions assayed here, P450c21 mRNA was very weakly expressed.

We further verified the presence of CYP2D4 and P450c21 proteins in rat brain using CYP2D4- and P450c21-specific antibodies. A Western blot analysis revealed high levels of CYP2D4 protein in cerebellum, striatum, pons, and medulla oblongata compared with other regions (Fig. 4A). The CYP2D4 content was calculated as 1.27, 0.97, 0.93, and 0.85 pmol/mg protein for cerebellum, striatum, pons, and medulla oblongata, respectively (Fig. 4B). Contents of CYP2D4 were well correlated to activity for bufuralol 1'2'-ethenylation, which is a CYP2D4-specific reaction (17), among the brain regions, and the correlation coefficient was calculated as $r = 0.861$ (Fig. 4C). In contrast, no band of P450c21 protein was detected in any rat brain region under conditions such that P450c21 protein from minute amounts of adrenal microsomes could be detected (Fig. 4A). These results demonstrated biochemically that CYP2D4 is widely distributed to

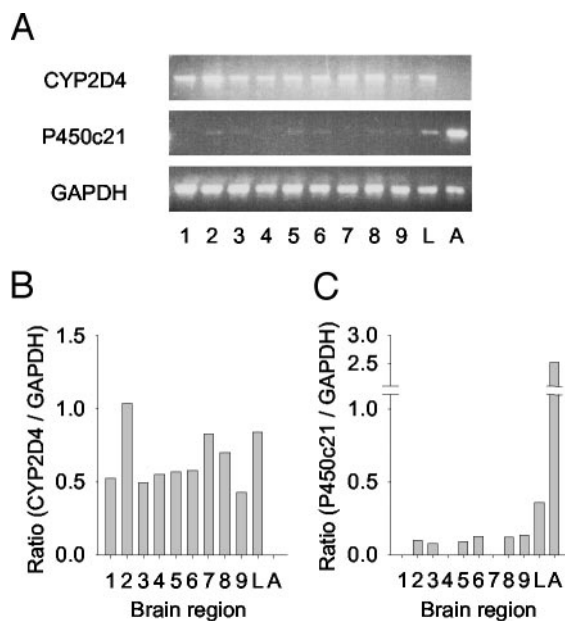


FIG. 3. Expression of CYP2D4 and P450c21 mRNAs in rat brain regions. Expression of mRNAs of CYP2D4 and P450c21 in rat brain, liver, and adrenal was estimated by RT-PCR (A) and expressed as the ratio of CYP2D4 (B) or P450c21 (C) to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Brain regions: lane 1, cerebrum; 2, cerebellum; 3, midbrain; 4, medulla oblongata; 5, thalamus; 6, hypothalamus; 7, hippocampus; 8, pons; 9, striatum. L, Liver; A, adrenal gland.

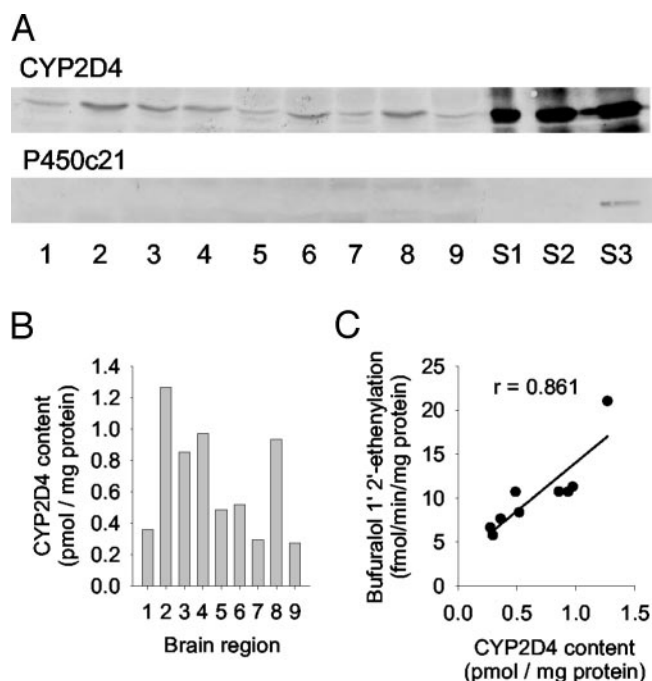


FIG. 4. Regional distribution of CYP2D4 protein in rat brain and its correlation to CYP2D4-specific metabolic activities. A, Levels of CYP2D4 (upper) and P450c21 (lower) protein in each rat brain region were determined by immunoblot analysis. Microsomes (50 μ g) from various rat brain regions (lane 1, cerebrum; 2, cerebellum; 3, midbrain; 4, medulla oblongata; 5, thalamus; 6, hypothalamus; 7, hippocampus; 8, pons; 9, striatum) were applied to a polyacrylamide gel, and immunoblotting was performed using anti-CYP2D4 or anti-P450c21 antibodies. Microsomes from recombinant CYP2D4 or anti-P450c21 antibodies. Microsomes from recombinant CYP2D4 (S1, 0.5 pmol; S2, 1 pmol; S3, 2 pmol) or adrenal gland (S1, 0.1 μ g; S2, 1 μ g; S3, 10 μ g) were also applied. B, CYP2D4 protein was quantitated based on the band density, using recombinant CYP2D4 protein as an authentic standard. C, Bufuralol (10 nmol) was incubated with microsomes from each rat brain region (0.5 mg) in a final volume of 0.5 ml 0.1 M potassium phosphate buffer (pH 7.4). The reaction was initiated by adding NADPH (0.5 μ mol).

all brain regions, especially cerebellum, striatum, and pons, and is more highly expressed than P450c21.

Allopregnanolone 21-hydroxylation by brain CYP2D4

Neurosteroids such as allopregnanolone promptly decrease brain excitability, acting as potent positive allosteric modulators of γ -aminobutyric acid (GABA) action at GABA_A receptors (24–26). Consequently, they have potential value in the treatment of various neuropsychiatric disorders (27, 28). Because allopregnanolone is a C-21 steroid, CYP2D4 might show 21-hydroxylation activity toward these GABA modulators. Among the CYP2D isoforms used here, only CYP2D4 and CYP2D6 showed 21-hydroxylation activity toward allopregnanolone (Fig. 5A), and also toward 5 α -dihydroprogesterone (data not shown). The K_m value of CYP2D4 is sufficient for *in vivo* reaction, *i.e.* $6.05 \pm 0.83 \mu$ M (Fig. 5B). Rat brain microsomal allopregnanolone 21-hydroxylation was not inhibited by anti-P450c21 antibodies, but was inhibited by quinidine and by anti-CYP2D4 antibodies (Fig. 5, C and D). Furthermore, fluoxetine, a representative selective serotonin reuptake inhibitor that is known to inhibit CYP2D activity (29), inhibited rat brain microsomal allopreg-

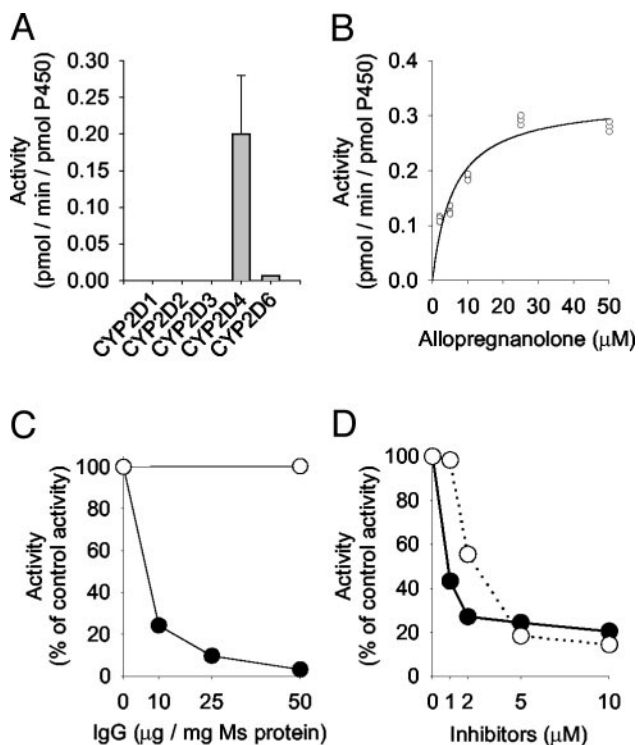


FIG. 5. Allopregnanolone 21-hydroxylation catalyzed by brain CYP2D isoforms. A, Recombinant microsomes from various CYP2D isoforms were incubated with 12.5 nmol allopregnanolone in a final volume of 0.5 ml 0.1 M potassium phosphate buffer (pH 7.4). Data are expressed as the mean \pm SD ($n = 3$). B, K_m values and V_{max} values were calculated by means of nonlinear regression: $6.05 \pm 0.83 \mu\text{M}$ and $0.33 \pm 0.016 \text{ pmol/min-pmol P450}$, respectively (mean \pm SD; $n = 3$). C, Rat brain microsomes (0.5 mg) were preincubated with various amounts of anti-CYP2D antibodies (filled circle) or anti-P450c21 antibodies (open circle) at room temperature for 15 min. After preincubation, 12.5 nmol allopregnanolone and 0.1 M potassium phosphate buffer (pH 7.4) were added to the mixture on ice. The reaction was initiated by adding NADPH (0.5 μmol). Allopregnanolone 21-hydroxylation activities were expressed as percentage of the control. D, Rat brain microsomes (0.5 mg) were incubated with 12.5 nmol allopregnanolone in the presence of various concentrations of the CYP2D-specific inhibitor quinidine (filled circle) or fluoxetine (open circle) in a final volume of 0.5 ml 0.1 M potassium phosphate buffer (pH 7.4).

nanolone 21-hydroxylation activity in a dose-dependent manner, and its IC_{50} value was estimated to be around 2 μM (Fig. 5D). These results raise the possibility that the brain CYP2D isoforms regulate levels of neurosteroids such as allopregnanolone, and that this regulation would be modified by central nervous system-active drugs such as fluoxetine.

Discussion

P450c21 mRNA was detected in the brain, although at approximately 100- to 10,000-fold concentration lower than that in the adrenal gland, by using quantitative RT-PCR methods (2, 3). Microsome-enriched fractions from human fetal tissues showed 21-hydroxylase activity, converting progesterone to 11-deoxycorticosterone (30). Several areas of the rat brain produced very small amounts of corticosterone and aldosterone from endogenous precursors through a synthetic cascade that must include 21-hydroxylase activity (31).

However, it has not been established that P450c21 enzyme is involved in steroid 21-hydroxylation in the brain.

In our studies, rat CYP2D4 and human CYP2D6, which are the predominant CYP2D isoforms in the brain (6–10), were shown to hydroxylate progesterone and 17 α -hydroxyprogesterone at the C-21 position. In rat brain microsomes, these progesterone and 17 α -hydroxyprogesterone 21-hydroxylation activities were not inhibited by anti-P450c21 antibodies, but were effectively inhibited by anti-CYP2D4 antibodies, indicating that these activities are due to CYP2D isoforms in the brain. P450c21 mRNA was detected in the brain using RT-PCR (2, 3), but the amount was very small. The immunohistochemical staining for P450c21 protein was observed in the thalamus and pons of rat (32). Indeed, our results confirmed that the expression of P450c21 mRNA and protein in the brain was extremely low compared with that of CYP2D4. These facts support our hypothesis that steroid 21-hydroxylation in the brain is mediated by CYP2Ds, not P450c21. However, the physiological relevance of the potential for adrenal steroid production in the brain is as yet unknown.

In addition to the function of CYP2D4 as a steroid 21-hydroxylase, CYP2D4 was revealed to metabolize neurosteroids such as allopregnanolone. Allopregnanolone is synthesized from progesterone via two sequential A-ring reductions. A 5 α -reductase first converts progesterone to 5 α -dihydroprogesterone, which is then further reduced by 3 α -hydroxysteroid oxidoreductase to form allopregnanolone. Enzymes involved in allopregnanolone formation were reported to be expressed in the brain (33, 34), and their distribution pattern is in good agreement with that of CYP2D4 (Figs. 3 and 4). Moreover, GABA $_A$ receptor (35) and glutamic acid decarboxylase (36), involved in GABA synthesis, are also highly expressed in CYP2D4-rich brain regions. These results strongly supported the view that CYP2D4 plays an important role in regulating brain levels of GABA modulators in cooperation with 5 α -reductase and 3 α -hydroxysteroid oxidoreductase.

Several recent studies have pointed to commonly used selective serotonin reuptake inhibitors as potential modulators of neurosteroid synthesis in the brain. Rat brain microsomal allopregnanolone 21-hydroxylation was inhibited not only by quinidine and anti-CYP2D4 antibodies, but also by fluoxetine, which increased the level of allopregnanolone in the brain of rats (37) and humans (38). However, the mechanism of this effect of fluoxetine is poorly understood: one report claimed that fluoxetine allosterically activates biosynthesis of allopregnanolone catalyzed by 3 α -hydroxysteroid oxidoreductase (39), whereas another study found no activation of 3 α -hydroxysteroid oxidoreductase (40). The concentration of fluoxetine that completely inhibits rat brain microsomal allopregnanolone 21-hydroxylation is approximately equivalent to the brain concentration of this drug at the dose used in the study that observed the fluoxetine-elicited increase of allopregnanolone (41). These results are consistent with the idea that the inhibition of CYP2D-mediated allopregnanolone degradation in the brain by fluoxetine caused the increase of allopregnanolone in the brain. Several other compounds, such as paroxetine and clozapine, also increase the brain allopregnanolone level *in vivo* (37, 42). It

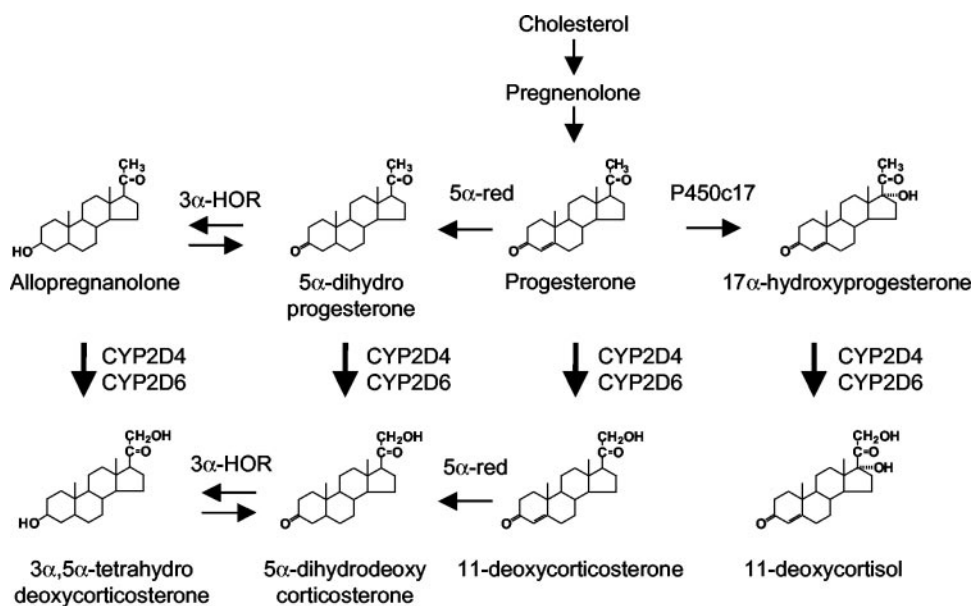


FIG. 6. Proposed scheme for involvement of CYP2D isoforms in the steroid synthesis in the brain.

will be intriguing to investigate the effects of other central nervous system-active drugs on brain neurosteroid levels, in relation to the affinity of the drugs for CYP2D.

In conclusion, our findings suggest that CYP2D isoforms, not P450c21, function as steroid 21-hydroxylase in the brain, and may positively regulate the levels of neurosteroids such as GABA modulators (Fig. 6).

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