Research report

Constitutive expression and localization of the major drug metabolizing enzyme, cytochrome P4502D in human brain

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Abstract

Cytochrome P4502D6, an important isoform of cytochrome P450, mediates the metabolism of several psychoactive drugs in liver. Quantitatively, liver is the major drug metabolizing organ, however metabolism of drugs in brain could modulate pharmacological and pharmacodynamic effects of psychoactive drugs at their site of action and explain some of the variation typically seen in patient population. We have measured cytochrome P450 content and examined constitutive expression of CYP2D mRNA and protein in human brain regions by reverse transcription polymerase chain reaction, Northern and immunoblotting and localized it by in situ hybridization and immunohistochemistry. CYP2D mRNA was expressed constitutively in neurons of cerebral cortex, Purkinje and granule cell layers of cerebellum, reticular neurons of midbrain and pyramidal neurons of CA1, CA2 and CA3 subfields of hippocampus. Immunoblot studies demonstrated the presence of cytochrome P4502D protein in cortex, cerebellum, midbrain, striatum and thalamus of human brain. Immunohistochemical localization showed the predominant presence of cytochrome P4502D not only in neuronal soma but also in dendrites of Purkinje and cortical neurons. These studies demonstrate constitutive expression of cytochrome P4502D in neuronal cell population in human brain, indicating its possible role in metabolism of psychoactive drugs directly at or near their site of action, in neurons, in human brain. © 2002 Elsevier Science B.V. All rights reserved.

1. Introduction

Cytochrome P450 (E.C. 1.14.14.1; P450) and associated monooxygenases are the principal class of drug metabolizing enzymes. This family of heme-protein is encoded by a supergene family, and the member proteins exist in multiple forms having distinct yet overlapping substrate specificities. Multiple forms of P450, which are selectively induced or inhibited by a variety of drugs, are known to exist in liver, the major organ involved in P450 mediated metabolism [7]. In the recent years, P450-mediated metabolism in extrahepatic organs (such as lung, kidney, skin, nasal epithelium) and the far-reaching consequences of such metabolism, in situ, within specific cells in target organs have been recognized in laboratory animals [10] and humans [20].

Psychoactive drugs such as antidepressants and neuroleptics are known to be metabolized by hepatic P450. The specific forms of liver P450 involved in the metabolism of some of these drugs have been characterized using rat and human liver microsomes. The major forms of P450 that have been demonstrated to play a predominant role in the

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metabolism of psychoactive drugs in both rat and human liver belong to P4503A, 2D, 2C and 1A families. P4502D6 is a constitutive form of hepatic P450 in human. Isoforms of P450 belonging to the 2D family are identifiable in rat and human liver where they mediate the metabolism of several commonly used psychoactive drugs, such as imipramine, amitryptaline and chlorimipramine [4,9] and haloperidol [34].

The hepatic metabolism of drugs and the genetic polymorphism exhibited by some forms of P450 are generally understood. These variables are reflected in the plasma levels of administered drugs. However, plasma drug levels often show poor correlation with therapeutic effect [13] suggesting that metabolism within the brain could influence the therapeutic outcome regardless of hepatic clearance and plasma drug levels. A moderate difference in the pharmacokinetics of psychoactive drugs often leads to dramatic pharmacodynamic effects, again suggesting that metabolism in situ within the brain could play a significant role [3].

Recent studies have shown metabolic activity of P450 isoforms in rat and human brain microsomes using specific substrates including some psychotropic drugs. These studies have demonstrated the biotransformation of imipramine to desipramine and hydroxyimipramine [31], and dextromethorphan to dextrorphan in rat brain microsomes [12]. The presence of dextromethorphan-*O*-demethylase activity in rat and human brain microsomes have also been confirmed by studies from other laboratories using specific inhibitors and antibodies to P4502D6 [36]. These studies have also demonstrated the low metabolic activity of P4502D6 in human brain microsomes and the need for addition of exogenous NADPH cytochrome P450 reductase for detecting the monooxygenase activity.

Over the past decade, our laboratory and others have demonstrated the presence of P450 mediated monooxygenase activities in rat and human brain and examined the metabolism of a variety of model substrates [27]. Of the several isoforms of P450 that are expressed constitutively in rat brain, P4502D4 is expressed in significantly higher amounts in brain as compared to other tissues including the liver [15]. In humans, the relative amounts of the different isoforms of P450 and their localization are as yet unclear. In view of the important role that P4502D plays in the metabolism of psychoactive drugs, we have investigated the expression of P4502D in human brain and localized the expression of this isoform of P450 using fluorescence in situ hybridization and immunohistochemistry.

2. Materials and methods

2.1. Materials

cDNA to CYP2D6 and antiserum to P4502D1 were obtained as gifts from Dr. J.P. Hardwick. DIG-RNA labeling and detection kit, anti-digoxigenin fab fragments linked to peroxidase and alkaline phosphatase were purchased from Boehringer Mannheim, USA. The tyramide signal amplification (indirect) kit for in situ hybridization was obtained from New England Nuclear, USA and Vectastain-ABC Elite kit was purchased from Vector Labs., USA. All other chemicals and reagents were of analytical grade and were obtained from Sigma (St. Louis, MO, USA) or Qualigens, India.

2.2. Animals

Male Wistar rats (3–4 months, 225–250 g) were obtained from the Central Animal Research Facility of NIMHANS. Animals had access to pelleted diet and water ad libitum. All animal experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. All efforts were made to minimize animal suffering, to reduce number of animals used and to utilize alternatives to in vivo techniques, if available.

2.3. postmortem studies

Rats were sacrificed by cervical dislocation and the animals were kept at 20 °C for different time periods (6, 12 and 24 h). At the end of each predetermined time period, animals were decapitated and liver and brain were removed rapidly. Control animals were sacrificed by cervical dislocation and the liver and brain removed immediately. The tissues were processed immediately for isolation of total RNA [6] or preparation of microsomes [26].

2.4. Studies with human brain

Human brains from traffic accident victims with no known neuro-psychiatric disorders were obtained from the Human Brain Tissue Repository, Department of Neuropathology, NIMHANS. Both male and female brains were used. The gender, age of the deceased and postmortem delay are given in Table 1. After autopsy, brains were washed in ice-cold saline and dissected into different regions such as cortex, hippocampus, striatum, midbrain, cerebellum and thalamus based on standard anatomical markings. All regions were flash frozen and stored at −70 °C immediately. Regions from each human brain was thawed on ice and used for the preparation of microsomes as described below. Microsomes were aliquoted and stored at −70 °C and used for assaying P450 content.

2.5. Preparation of microsomes

All steps were performed at 4 °C. Rat brain or human brain tissue was homogenized using a Potter–Elvehjem homogenizer in nine volumes of ice-cold Tris buffer (0.1 M, pH 7.4) containing dithiothreitol (0.1 mM), EDTA (0.1

Table 1
Specific content of P450 in human brain regions

Subject	Gender	Age (years)	Postmortem delay (h)	Specific content (nmole P450/mg protein)			
				Cortex	Cerebellum	Striatum	Thalamus
I	Female	8	8	0.036	0.043	0.024	0.034
II	Male	65	6	0.023	0.022	0.025	0.028
III	Female	28	12	0.020	0.021	0.028	0.031
Mean±S.D.				0.026±0.008	0.031±0.01	0.026±0.002	0.031±0.003

Cytochrome P450 content was measured from the reduced minus oxidized carbon monoxide binding spectrum in human brain regions obtained at autopsy from three subjects. Values are average of two measurements performed using two different concentrations of microsomal protein.

mM), KCl (1.15%, w/v), phenylmethylsulfonyl fluoride (0.1 mM), butylated hydroxytoluene (22 μM) and glycerol (20%, v/v), previously bubbled with nitrogen (buffer A). The homogenate was centrifuged at 17,000 g for 30 min at 4 °C. Thereafter, the supernatant was centrifuged at 100,000 g for 1 h at 4 °C to give the microsomal pellet [26]. The pellet was washed, suspended in a small volume of buffer A, aliquoted and stored at −70 °C. Protein concentration was measured by a dye-binding method [2]. Total P450 content was measured from the carbon monoxide reduced minus oxidized difference spectrum [18].

2.6. Immunoblot analysis

Microsomal protein from human brain regions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [16]. Proteins were transferred from the gel to nitrocellulose paper [33] and incubated with the antiserum to rat liver P4502D1, followed by incubation with anti-rabbit IgG labeled with alkaline phosphatase. The immunostained bands were detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as chromogenic substrates for alkaline phosphatase.

2.7. Northern analysis and fluorescence in situ hybridization

The cDNA to CYP2D6 provided by Dr. James Hardwick [8] was subcloned into pBluescript, verified by DNA sequencing and used for preparation of riboprobes. Total RNA from rat liver, brain and human frontal cortex was extracted as described by Chomezynski [6]. Total RNA was separated electrophoretically and transferred onto positively charged nylon membranes by capillary transfer [14]. After UV crosslinking, the membranes were hybridized overnight at 55 °C with the digoxigenin-labeled antisense riboprobe prepared using the cDNA to CYP2D6, washed, incubated with antibody to digoxigenin fab fragments conjugated with alkaline phosphatase. The bands were visualized using nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate as chromogenic substrates for alkaline phosphatase.

Regions from human brain (cortex, cerebellum, midbrain and hippocampus) were dissected out and fixed in buffered paraformaldehyde. The tissue was processed for paraffin embedding, serial sections (8–10 μm thick) were cut under RNase-free conditions. Sections were dewaxed, hydrated in graded ethanol, acetylated and treated with proteinase K (100 μg/ml) for 20 min at 37 °C. The sections were rinsed in phosphate-buffered saline (PBS) and dehydrated using graded ethanol. Digoxigenin-labeled sense (for control sections) and antisense cRNA probes were synthesized from cDNA to CYP2D6 using T3 and T7 RNA polymerases. Sections were hybridized overnight at 55 °C with the sense or antisense probes. After hybridization, the sections were washed, blocked with 0.5% (w/v) bovine serum albumin (NEN Life Sciences Products, USA) and incubated with antibody to digoxigenin fab fragments conjugated to horseradish peroxidase. After washing, the sections were incubated with biotinylated tyramide (NEN Life Sciences Products) followed by FITC labeled streptavidin. Finally the sections were washed, dried and mounted prior to examination under a fluorescence microscope.

2.8. Reverse transcription polymerase chain reaction (RT-PCR) analysis of mRNA from human brain cortex

Total RNA was isolated from frontal cortex of autopsy human brain samples as described above. The first strand of cDNA was synthesized using 1 μg of total RNA from human brain, oligo dT primers and reverse transcriptase. The second strand was synthesized using T4 DNA polymerase. The double-stranded DNA was purified by phenol/chloroform extraction and alcohol precipitation. The primer sequences, nucleotide location, expected product size and Genebank accession numbers for CYP2D6 and β-actin are as follows: The primers used for CYP2D6 (Genebank accession number=M20403) analysis were 5′ to 3′-TGATGAGAACCTGCGCATAG (representing bases from 1207 to 1186) and 3′ to 5′-ACCGAT-GACAGGTTGGTGAT (representing bases from 875 to 892) and the expected size of the PCR product is 333 bp. The primers used for β-actin (Genebank accession number=X00351) were 5′ to 3′-TGACGGGGTCAC-

CCACACTGTGCCCATCTA (representing bases from 1038 to 1067) and 3′ to 5′-CTAGAAGCATTGCGGT-GGACGATGGAGGG (representing bases from 1876 to 1905) and the expected PCR product size is 661 bp [23]. RT-PCR analysis of β-actin was carried out simultaneously as a positive control. The cDNA synthesized from 1 μg of RNA was suspended in 5 μl of 10 mM Tris–HCl (pH 8.3) and added to the final reaction mixture which contained 50 mM KCl, 200 μm of each dNTP, $MgCl_2$ (2.5–3.5 mM), primers (80–85 pmole) and 0.25 units of AmpliTaq DNA polymerase. The following thermal cycling conditions were used for β-actin, initial denaturation at 94 °C for 5 min followed by 40 cycles consisting of 48 s at 94 °C, 48 s at 68 °C and 1.5 min at 72 °C with a final extension at 72 °C for 10 min. The thermal cycling conditions used for CYP2D6 were as follows: initial denaturation at 94 °C for 5 min followed by 40 cycles consisting of 30 s at 94 °C, 30 s at 68 °C and 60 s at 72 °C with a final extension at 72 °C for 2 min. The negative control consisted of sterile water instead of template DNA. The PCR products (20 μl) were separated by electrophoresis using 1.2% agarose gel and stained with ethidium bromide. The identity of the PCR products was confirmed by Southern blotting using digoxigenin labeled cDNA to CYP2D6 and sequencing.

2.9. Immunohistochemical studies

Human brain obtained at autopsy was dissected out and fixed in 4% (w/v) buffered paraformaldehyde. The tissue was processed for paraffin embedding and serial sections (8–10 μm thick) were cut. Sections were dewaxed by heating at 70 °C, followed by xylene treatment and brief wash in chloroform to remove residual xylene. To block endogenous peroxidase reaction, the sections were exposed to methanol containing hydrogen peroxide (3%, v/v) for 30 min. They were hydrated in graded alcohol followed by brief wash in water and phosphate buffered saline (10 mM, pH 7.4, PBS). The sections were placed in metal racks and lowered into the boiling sodium citrate buffer (pH 6.8) and pressure cooked for 10 min. After cooling, the slides were transferred to PBS. To minimize the binding of non-specific proteins, sections were incubated in normal goat serum for 30 min at room temperature. The sections were then washed in PBS and incubated with antiserum to P4502D1 (diluted to 1:1000 in PBS) at 37 °C for 2 h. After washing, they were incubated in biotinylated anti-rabbit IgG solution (1:500 dilution) for 1 h at room temperature.

Fig. 2. Dithionite reduced carbon monoxide binding spectrum of microsomal P450 from human brain cortex and cerebellum. (A) Microsomes from cortex region of human brain from subject I was used at a protein concentration of 0.25 mg/ml. The specific content of P450 in the microsomes was estimated to be 0.034 nmole of P450/mg protein. (B) Dithionite reduced carbon monoxide binding spectrum of human brain cerebellum (subject I) is depicted. The protein concentration was 0.25 mg/ml and the specific content of P450 was estimated to be 0.043 nmole of P450/mg protein.
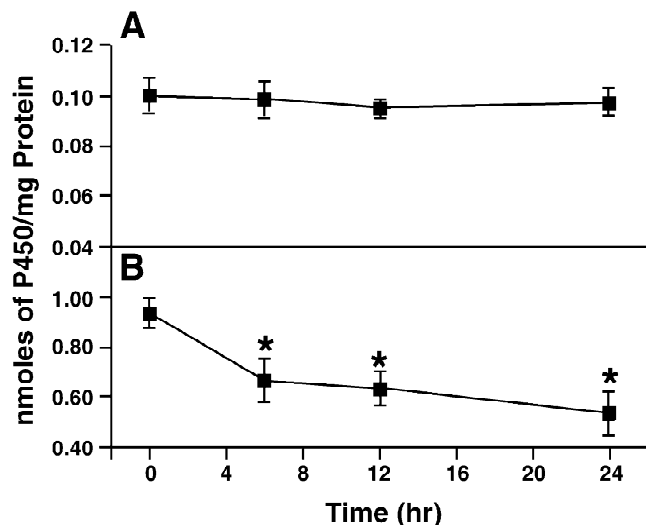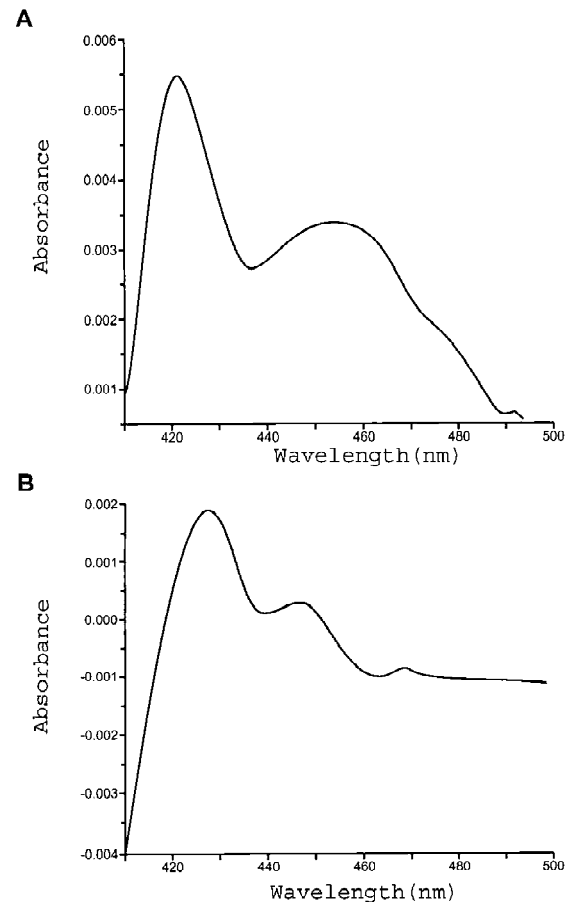

Fig. 1. Effect of postmortem delay on P450 content in rat brain and liver. P450 content was estimated in microsomes prepared from (A) whole brain or (B) liver of rats sacrificed after various periods of postmortem delay (6, 12 and 24 h). Values are mean±S.D. (n=3 animals). Asterisks indicate the values that are significantly different from controls ($P<0.05$).
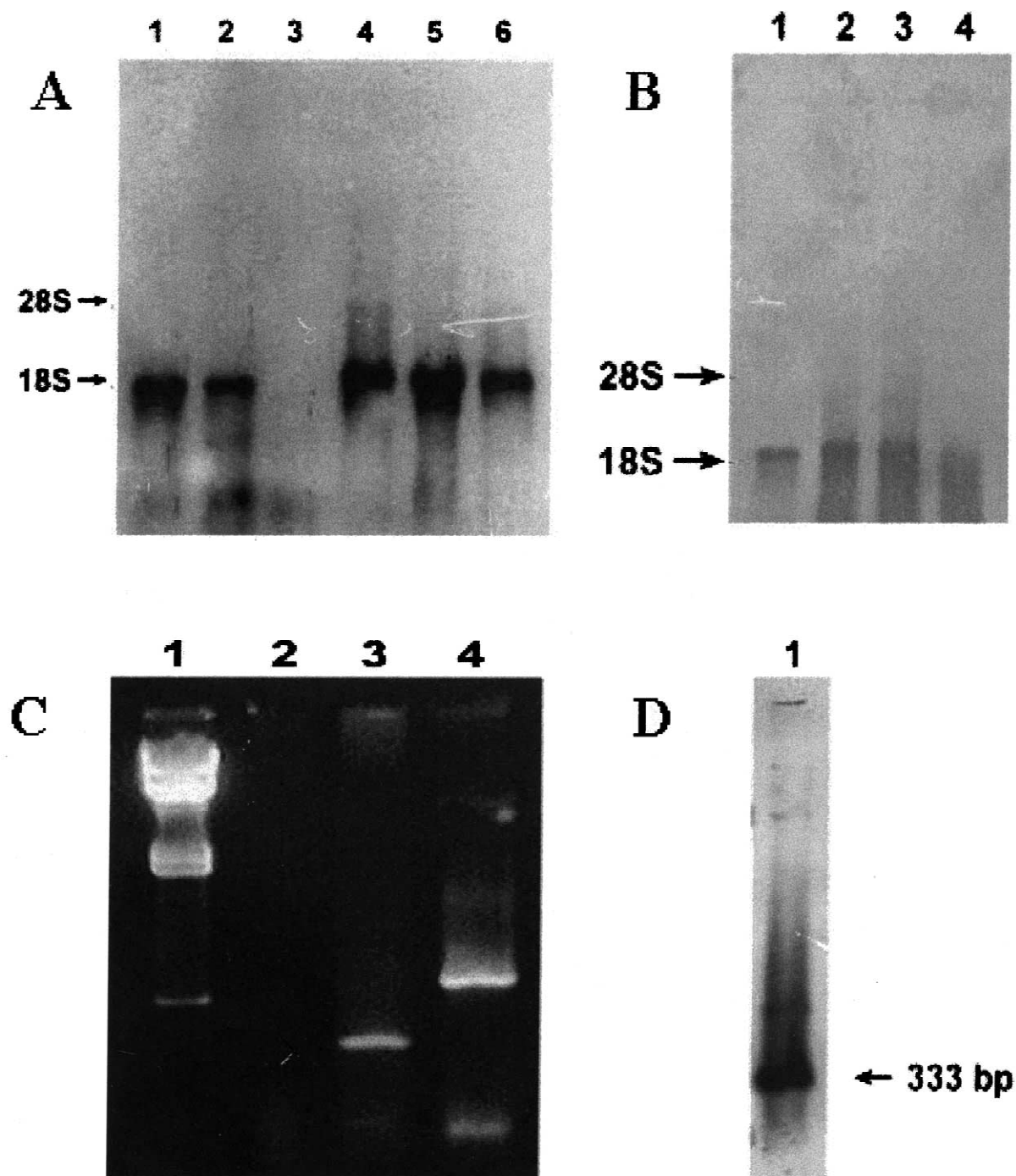
Fig. 3. Northern blot analysis of (A) effect of postmortem delay on CYP2D mRNA in rat brain and liver and (B) human brain total RNA using cDNA to CYP2D6. (A) Total RNA was extracted from liver (lanes 1, 2 and 3; 5 μg each) and whole brain (lanes 4, 5 and 6; 12 μg each) of rats sacrificed after various periods of postmortem delay (0, 12 and 24 h) and subjected to Northern blot analysis. The expression of rat liver and brain CYP2D mRNA was seen as band at approximately 1.6 Kb. (B) Total RNA from rat liver (lane 1, 10 μg) and human brain cortex (lanes 2–4, 15 μg) were electrophoresed under denaturing conditions. After transfer to nylon membrane, the blots were hybridized with antisense riboprobe prepared using cDNA to CYP2D6. The total RNA was prepared from cortex of human brain obtained from subjects 1, 2, 3 (lanes 2, 3, 4, respectively). The mobility of the 18S and 28S ribosomal RNA is indicated The expression of human brain CYP2D mRNA was seen as a band at approximately 1.6 Kb. (C) Analysis of constitutive expression of CYP2D6 mRNA by RT-PCR. Total RNA was extracted from autopsy human brain cortex. RT-PCR reaction was carried out using primers specific to β-actin (lane 4) and CYP2D6 isoform (lane 3). Control reaction was performed without template DNA (lane 2). PCR amplified DNA was by agarose gel electrophoresis and stained with ethidium bromide. The size of the PCR products were 661 bp (for β-actin) and 333 bp for CYP2D6. (D) The PCR amplified DNA of 333 bp hybridized with digoxigenin labelled cDNA to CYP2D6 as examined by Southern blotting indicating the homology of the amplified PCR product with CYP2D6.

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After further washing, they were incubated with VECTAS-TAIN Elite ABC reagent for 30 min at room temperature. The sections were then washed briefly in PBS followed by water. The color was developed using diaminobenzidine. The sections were finally washed with water, dehydrated in graded ethanol, cleared with xylene, air dried and mounted using Permont.

3. Results

3.1. Effect of postmortem delay on specific content of P450 in rat brain and liver

Total P450 content was estimated in brain microsomes of rats sacrificed after various time periods of postmortem
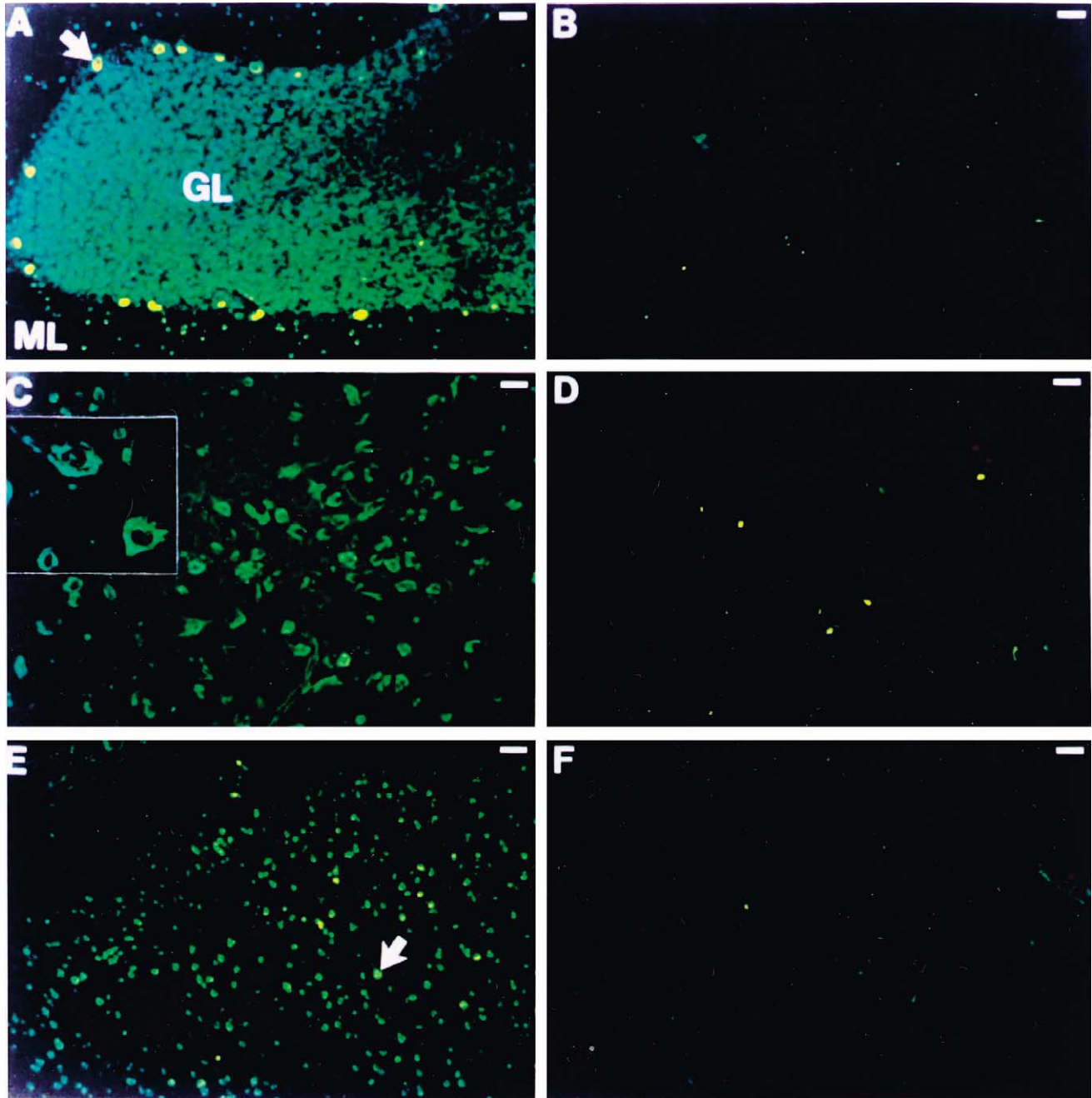

Fig. 4. Localization of CYP2D mRNA in human brain by fluorescence in situ hybridization (A) Fluorescent labeling of the Purkinje cells (arrow) and the granule cell layer (GL) in human cerebellum indicating the presence of CYP2D mRNA. Sparse staining of the cells in the molecular layer (ML) was also noted. (B) No staining was seen in section hybridized with the sense probe. Bar=200 μm. (C) Reticular neurons in the midbrain expressed CYP2D mRNA. Inset: Higher magnification of giant reticular neuron. (D) Control section of midbrain hybridized with the sense probe. Bar=200 μm. (E) Intense fluorescence was seen in neuronal cell layers of the human brain frontal cortex. Differential fluorescence was seen delineating the laminar architecture of the cortex (F) Control section hybridized with the sense probe. Bar=200 μm.

delay. As shown in Fig. 1A, no significant change in brain microsomal P450 content was observed even after 24 h of postmortem delay. The specific content of brain P450 varied from 0.09 to 0.1 nmole of P450/mg protein during the period of examination. In contrast, liver P450 content decreased from 0.93 nmole of P450/mg protein to 0.53 nmole of P450/mg protein during 24 h of postmortem delay (Fig. 1B). In fact within 6 h, there was 29% loss in liver microsomal P450 content. These studies indicate the relative stability of brain microsomal P450 to autolytic changes that are known to occur during postmortem. The studies on human brain P450 were hence carried out using human autopsy tissues that were obtained within 12 h of postmortem delay.
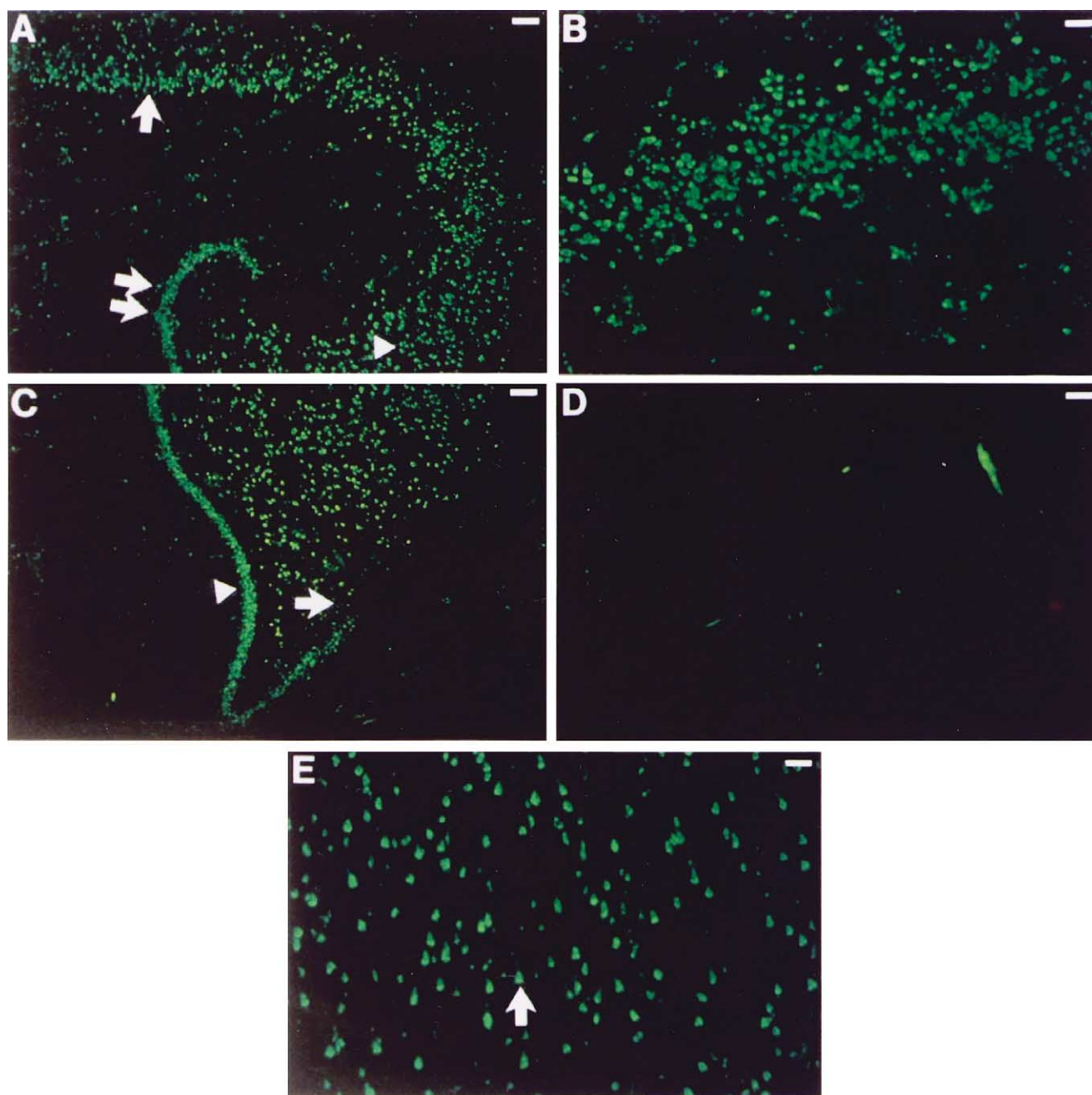

Fig. 5. Localization of CYP2D mRNA in human brain hippocampus using fluorescence in situ hybridization. (A) The presence of CYP2D mRNA was seen in the pyramidal neurons of CA2 subfield in the hippocampus (arrow). A portion of the dentate gyrus is also seen (double arrow). Bar=400 μm. (B) Higher magnification of CA2 pyramidal cell layer hippocampus from human brain. Bar=200 μm. (C) Localization of CYP2D mRNA in the granule cell layer of dentate gyrus (arrow head), CA3 subfield and in the interneurons of hilius (arrow). Bar=400 μm. A similar section hybridized with the sense probe did not show any fluorescence (D). Bar=200 μm. (E) The presence of CYP2D mRNA was observed in the CA1 pyramidal neurons (arrow). Bar=200 μm.
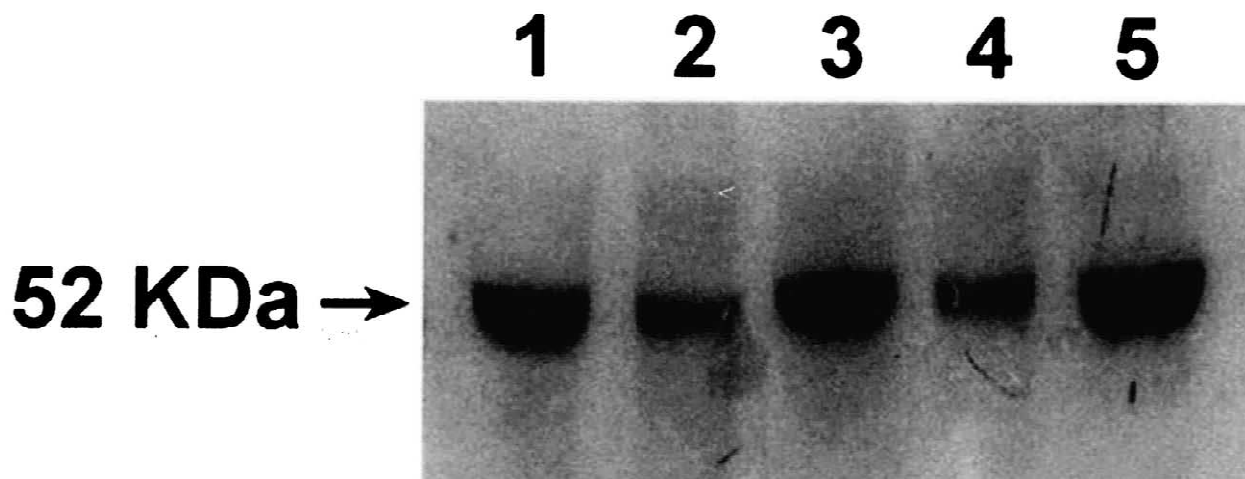
Fig. 6. Immunoblot analysis of microsomal protein from human brain regions using antiserum to rat liver P4502D1. Microsomal protein from brain regions obtained at autopsy from subject 1 were subjected to SDS–PAGE and transferred to nitrocellulose membrane. The blots were immunostained with antiserum to P4502D1. Immunostained bands were detected at 52 kDa. The lanes contained 50 μg of microsomal protein from cerebral cortex (lane 1), cerebellum (lane 2), midbrain (lane 3), striatum (lane 4) and thalamus (lane 5).

3.2. Cytochrome P450 content in human brain regions

Specific content of P450 was estimated in the brain regions of autopsy tissue obtained from three subjects. The gender, age and time interval between death and autopsy are given in Table 1. The carbon monoxide reduced minus oxidized spectrum of human brain microsomes from cortex and cerebellum exhibited a characteristic absorption spectrum with two peaks at 450 and 425 nm (Fig. 2). The peak at 450 nm represents P450. The specific content of P450 was calculated by measuring the absorbance at 450 nm. P450 measurements varied from 0.02 to 0.04 nmole of P450/mg protein in different regions of the brain from the three subjects (Table 1). We were able to procure only four regions, namely, cortex, cerebellum, striatum and thalamus for these experiments. P450 content in cerebellum from subject 1 was twofold of that in the striatum. However, this pattern of distribution was not seen in the other two brains that were examined.

### 3.3. Effect of postmortem delay on stability of CYP2D6 mRNA in rat brain and liver

Total RNA was extracted from liver and whole brain of rats subjected to various time periods of postmortem delay. As shown in Fig. 3A, degradation of CYP2D6 mRNA was apparent in liver after 12 h of postmortem delay and no band was detectable after 24 h of postmortem delay. In contrast, in the brain, CYP2D6 mRNA levels did not change significantly up to 12 h postmortem delay and degradation was seen only after 24 h of postmortem delay. This study indicates the relative stability of brain mRNA to autolytic changes that are known to occur during postmortem.

3.4. Northern blot and RT-PCR analysis of CYP2D expression in human brain

Northern blot analysis of total RNA from human brain cortex using the cDNA to CYP2D6 revealed the constitutive expression CYP2D mRNA. The molecular mass of the transcript was approximately 1.6 Kb which was similar to that seen in rat liver (Fig. 3B). In all the three brain samples the expression of the full-length mRNA to CYP2D was observed. RT-PCR analysis of the cDNA prepared from human brain RNA yielded the expected 333 bp PCR amplified product, confirming the constitutive expression of cytochrome P4502D in the human brain (Fig. 3C). RT-PCR analysis of β-actin which was carried out simultaneously as a positive control yielded the expected product of 661 bp (Fig. 3C). The 333 bp RT-PCR product was subjected to Southern blotting using the digoxigenin labeled cDNA to CYP2D6 and the hybridized band was detected at 333 bp, indicating that the amplified product shared homology with CYP2D6 (Fig. 3D). The

Fig. 7. Immunohistochemical localization of P4502D in human brain cerebellum and CA1 area of hippocampus using antiserum to P4502D1. (A) Localization of 4502D in the granule cells (arrow) and Purkinje cell (double arrow) is depicted. Immunostaining was seen in the plasma membrane as indicated by the arrowhead. Bar=10 μm. (B) Control section incubated with non-immune serum did not show immunostaining in the cerebellum. Bar=100 μm. (C) Immunostaining of CA1 pyramidal neurons indicating the presence of P4502D. Bar=10 μm. (D) Similar section treated with non-immune serum did not show immunostaining of the CA1 neurons. Bar=100 μm.
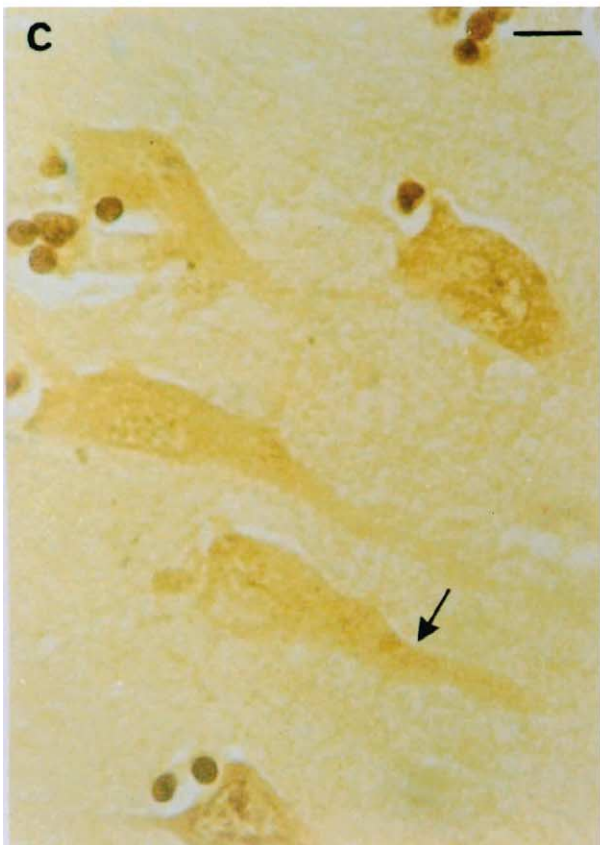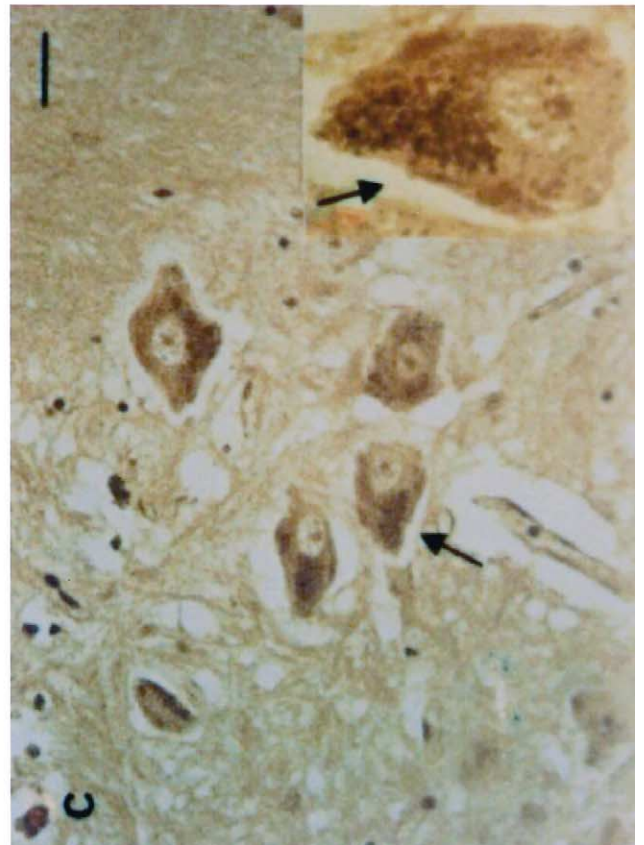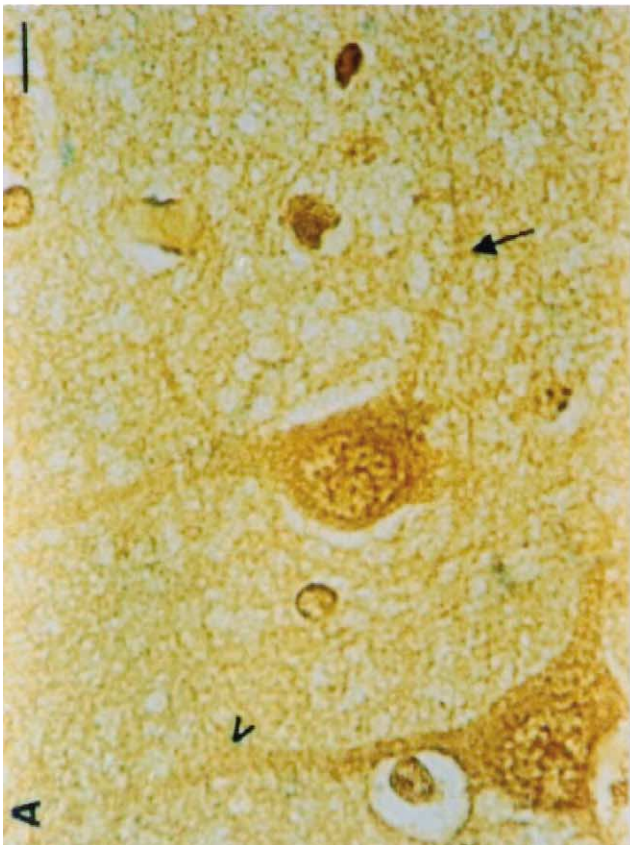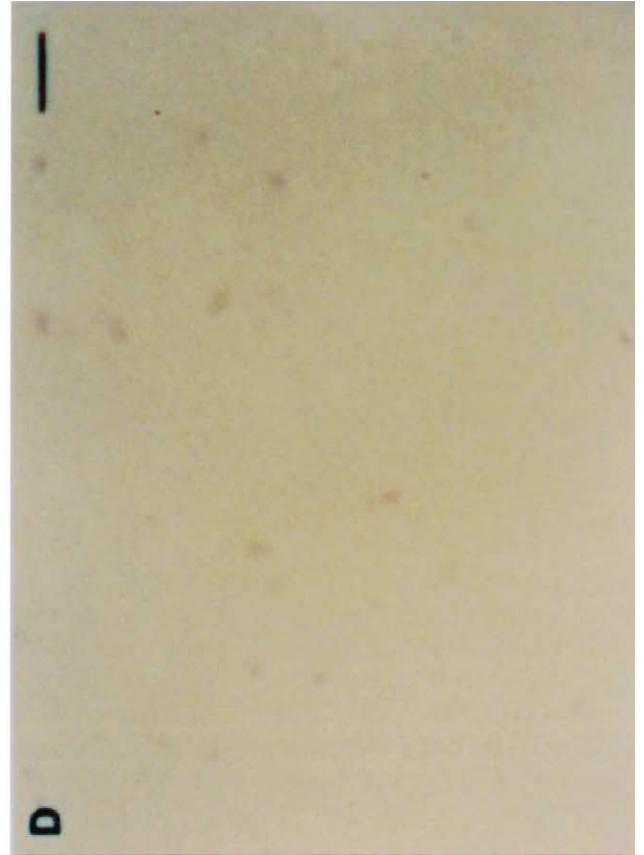
sequencing of the RT-PCR product revealed the complete sequence identity with CYP2D6 in the amplified region.

3.5. Localization of CYP2D mRNA in human brain by fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) studies demonstrated the presence of CYP2D mRNA predominantly in neuronal cells in human brain regions. In the cerebellum, Purkinje cells and granule cell layer showed significant fluorescence with the granule cell layer being more intensely stained than the Purkinje cell (Fig. 4A). Occasional staining was seen in the molecular layer. In the midbrain, reticular neurons were stained although not as intensely as seen in the cerebellum and cortex (Fig. 4C). High levels of CYP2D mRNA was seen in neuronal cells in the cerebral cortex (Fig. 4E) while sections hybridized with the sense probe showed no fluorescence (Fig. 4F). Intense fluorescence was seen in the hippocampus (Fig. 5), in the pyramidal neurons of CA1, CA2, CA3 subfields and in the granule cell layer of dentate gyrus (Fig. 5C), while similar sections hybridized with the digoxigenin labeled sense probe showed no fluorescence (Fig. 5D). The pyramidal neurons in CA1 showed intense cytosolic staining (Fig. 5E). The neurons in CA2 were also labeled and the compact CA2 pyramidal cell layer was clearly discernable (Fig. 5B). In CA3 also the pyramidal neurons were intensely fluorescent (Fig. 5C), along with the interneurons of the hilius.

3.6. Immunoblot studies

Immunoblot analysis of microsomes prepared from different regions such as, cortex, cerebellum, midbrain, striatum and thalamus of human brain obtained at autopsy from subject 2 using antiserum to rat liver P4502D1 revealed the presence of a immunoreactive protein of molecular mass 52 kDa, thus demonstrating the constitutive expression of P4502D6 protein in various regions of human brain (Fig. 6).

3.7. Localization of P4502D by immunohistochemistry

Immunohistochemical analysis using antiserum to rat liver P4502D1demonstrated the predominant presence of P4502D in neuronal population in concordance with the in situ experiments. In the cerebellum, granule cells and Purkinje cells were stained (Fig. 7A), with the granule cells being more intensely stained compared to Purkinje

cells, similar to the observation made in the in situ studies. In the Purkinje cell, in addition to the intense punctate staining seen in the neuronal cell body, the dendritic arborization and synaptic terminals were also significantly stained. The CA1 neurons in the hippocampus were also stained although less intensely than in the cerebellum (Fig. 7C). Intense staining of the neuronal cell body and apical dendrites was seen in frontal cortex (Fig. 8A). Immunostaining was also observed in the synaptic terminals in a manner similar to that seen in Purkinje cells (Fig. 7A). Reticular neurons of the midbrain (Fig. 8C) were also immunostained. Similar sections incubated with non-immune serum did not show any staining.

4. Discussion

The studies on effect of postmortem delay on CYP2D mRNA and P450 content in rat brain revealed that the brain CYP2D mRNA was relatively stable to autolytic changes that normally occur during postmortem delay. The P450 content in brain did not change significantly up to 24 h after death in contrast to liver where significant loss of activity was seen after 6 h. In the present study, we estimated P450 content in human autopsy brains that were obtained within 12 h of postmortem delay. Presence of functional P450 in the autopsy brain was confirmed in agreement with our earlier observations [1]. Northern blot analyses for determining the expression of CYP2D6 was carried out using the same brain tissue samples. Constitutive expression of CYP2D was detectable in human brain as illustrated in Fig. 3B. The expression of CYP2D in human brain had been examined earlier by using the polymerase chain reaction to amplify cDNA from a human caudate lambda gt11 library encoding exons 6–9 of the human CYP2D6 gene [35]. However, the present study provides the first evidence for the presence of the full-length CYP2D mRNA of 1.6 Kb in human brain. CYP2D is expressed relatively abundant in human brain as evidenced from the northern blot (Fig. 3B). In humans lacking the CYP2D6 gene (a known genetic polymorphism), both the northern and in situ hybridization studies would have yielded negative results. However, none of the human brain samples used in the present study were polymorphic for the absence of the CYP2D6 gene.

The localization of P4502D mRNA and protein in human brain using in situ hybridization and immunohistochemistry demonstrated the predominant presence of the enzyme in the neuronal cell population in the cortex,

Fig. 8. Immunohistochemical localization of P4502D in human brain frontal cortex and midbrain using antiserum to P4502D1. (A) Intense immunolabeling of neurons in frontal cortex was seen indicating the presence of P4502D. The arrow shows the immunostaining of the dendrites. Bar=10 μm. (B) No staining was seen in cerebral cortex section incubated with non-immune serum. Bar=100 μm. (C) Immunostaining of reticular neurons (arrow; also shown in higher magnification in the inset) in the midbrain indicating the presence of P4502D was observed. Bar=25 μm. (D) A similar section of midbrain treated with non-immune serum did not show immunostaining. Bar=100 μm.

Purkinje and granule cell layer of the cerebellum, granule cell layer and pyramidal cells of the hippocampus. While the in situ hybridization studies provided the specific localization of the CYP2D mRNA (Figs. 4 and 5), immunohistochemistry delineated the sub-cellular localization of the P4502D protein within the neuronal cells (Figs. 6 and 7).

A close association between the dopamine transporter and P4502D1 has been observed in membrane preparation from striatum [25]. Further the possible presence of P4502D in the synaptic terminals in close association with opiate receptors has also been postulated [29,30]. However, definitive evidence for the above has not been presented so far. The immunohistochemical evidence shown in the present study demonstrates the presence of P4502D in the synaptic terminals in the Purkinje neurons in cerebellum as well as neurons in cerebral cortex. The predominant presence of P4502D seen in the synaptic terminals in the present study was not a common feature of subcellular distribution of other isoforms of P450 (such as P4501A1, 3A and 2B) in the human brain as studied by immunohistochemistry in our laboratory (data not shown). These observations hence point to a role for P4502D in the synaptic terminal. It is also to be mentioned that P4502D is the major form of P450 in brain [15]. More recent studies on hepatic P4502D6 have also provided evidence for the presence of P4502D6 in the plasma membrane [17,24]. However, the presence of this isoform of P450 in plasma membrane of neurons assumes greater significance since this points to the fact that not only is P4502D localized in the neurons (where the psychoactive drugs act) but also in the same sub-cellular compartment where the primary interaction between the drugs and the neurotransmitter receptors/transporters takes place.

Recent studies have also demonstrated that recombinant rat cytochrome P450 2D1, 2D4 and 2D6 possess progesterone 6â and 16á-hydroxylation activities. These activities were completely inhibited when the incubations were carried out in presence of the antisera to the respective P450 isoforms [11]. These findings support the idea that isoforms of cytochrome P450 belonging to the 2D family are involved in the metabolism of both xenobiotics and the endogenous neuroactive steroids, such as progesterone and its derivatives, in brain tissues.

The expression of CYP2D in human brain neurons is also important since P4502D could potentially play a role in the metabolism of environmental chemicals to detoxified metabolites. These detoxification pathways have far-reaching implications since a certain degree of association seems to exist between the incidence of Parkinson's disease and genetic polymorphism of P4502D6 seen in human subjects [28,32]. While this observation arguably has been confirmed in a large population through genotyping [19], there are other reports indicating that such an association may not exist [26]. Nevertheless, it is generally noted that the incidence of Parkinson's disease is associ-ated with both genetic susceptibility and environmental factors such as exposure to pesticides [5,21,22]. Thus, the xenobiotic detoxifying capability of the brain could potentially play an important role in predisposition to certain neurodegenerative diseases such as Parkinson's disease.

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