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Research report

Presence of splice variant forms of cytochrome P4502D1 in rat brain but not in liver

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Abstract

Cytochromes P450 (P450), a family of heme-containing proteins, is involved in the oxidative metabolism of both foreign and endogenous compounds. Although liver is quantitatively the major organ involved in the metabolism of most xenobiotics, there is increasing evidence that these enzymes are present in extrahepatic tissues, such as lung, kidney, brain, etc and they may contribute to the in situ metabolism of xenobiotics in these organs. The possible relationship between genetic polymorphism seen in P4502D6 and incidence of neurodegenerative diseases, such as Parkinson's disease, has prompted the characterization of P4502D enzymes in rat brain. In the present study, we demonstrate that P4502D1 (the rat homologue of human P4502D6) is constitutively expressed in rat brain and the mRNA and protein are localized predominantly in neuronal cell population in the olfactory bulb, cortex, cerebellum, and hippocampus. An alternate spliced transcript of CYP2D1 having exon 3 deletion was detected in rat brain but not in liver. Deletion of exon 3 causes frame shift and generates a stop codon at 391 bp relative to the start codon ATG leading to premature termination of translation. Thus, Northern blotting and in situ hybridization represent contributions from functional transcripts and alternate spliced variants that do not translate into functional protein. Further, the splice variant having partial inclusion of intron 6 detected in human brain was not detected in rat brain indicating that alternate spliced gene products of P450 enzymes are generated in species-specific and tissue-specific manner.

Theme: Disorders of nervous system
Topic: Neuropsychiatric disorders

Keywords: Brain; Drug metabolism; Cytochrome P450; CYP2D; Monooxygenase

1. Introduction

Cytochrome P450 (E.C. 1.14.14.1; P450) and associated monooxygenases, a family of heme proteins, are the principal class of drug metabolizing enzymes. They are 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 [8]. In recent years, there is increasing evidence that these enzymes are present in extrahepatic tissues such as lung, kidney, and brain and that they may contribute to the metabolism of drugs and activation of carcinogens and toxins in situ in the target tissue [3,13,23]. P450-mediated metabolism of psychoactive drugs directly in the brain can lead to local pharmacological modulation at the site of action and result in variable drug response. The inter-individual variability in hepatic metabolism of drugs

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caused by genetic polymorphism exhibited by some forms of P450, such as P4502D6, is reflected in the plasma levels of administered drugs. But plasma drug levels often show poor correlation with therapeutic effect [14] 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 suggesting that metabolism in situ within the brain could play a significant role [5].

Over the past decade, studies from our laboratory and others have demonstrated the presence of a competent microsomal P450 system in the rodent [1,13,34] and human [23,29] brain and its ability to metabolize a variety of xenobiotics. The appearance of multiple forms of P450 in brain and their selective inducibility by a variety of drugs and xenobiotics has also been identified [1,2,33,36]. Significant differences are seen in the regulation and function brain P450 enzymes compared to liver [27,35,37]. For example, drugs, such as alprazolam, are metabolized variably in liver and brain wherein relatively larger amount of the active metabolite is generated in rat brain compared to liver [27]. These observations have indicated the possible existence of unique P450 isoforms in brain that are different from the well-characterized hepatic P450s.

CYP2D is one of the major forms of P450 present in both rat [17] and human brain [6]. In rats, five genes belonging to the CYP2D family (CYP2D1, CYP2D2, CYP2D3, CYP2D4, CYP2D5) have been described [9,15,25], whereas in humans, one gene (CYP2D6) and several pseudogenes (CYP2D7 and CYP2D8) are known [24]. In humans, 5–10% of Caucasians exhibit defects in CYP2D6 alleles with resultant decreased rates of metabolism of CYP2D6 substrates [10,11]. Sprague–Dawley rats have a variant 2D1 allele, 2D1v, whereas Dark Agouti rats have no detectable expression of 2D1 mRNA in the liver [21].

Although CYP2D6 mRNA is mainly expressed in human liver, it has also been detected in human brain [32];

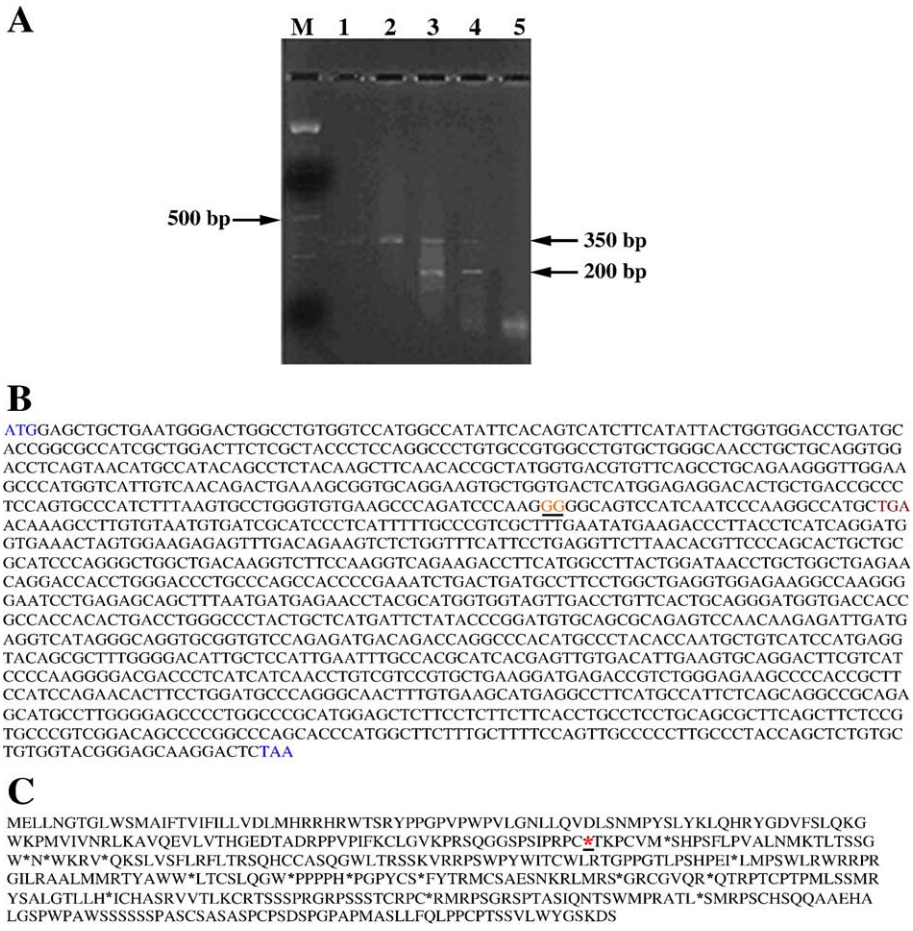


Fig. 1. Analysis of presence of exon 3 deletion in rat brain by RT-PCR. (A) RT-PCR analyses using specific primers for detecting the presence of exon 3 deleted splice variants in rat brain shows the presence of the splice variant form in rat brain. Lanes 1 and 2—RT-PCR product (approximately 350 bp) using rat liver cDNA. Lanes 3 and 4—PCR amplified product obtained using rat whole brain cDNA showing the presence of additional lower band (200 bp). Lane 5—negative control performed without DNA template. 'M' represents 0.5 Kb DNA ladder. (B) The mRNA sequence of CYP2D1 constructed from the sequence data of the 200 bp RT-PCR product as depicted above. (C) The translated protein sequence of the exon 3 deleted form of rat brain showing the premature termination of translation at amino acid 131 (represented by asterisk).

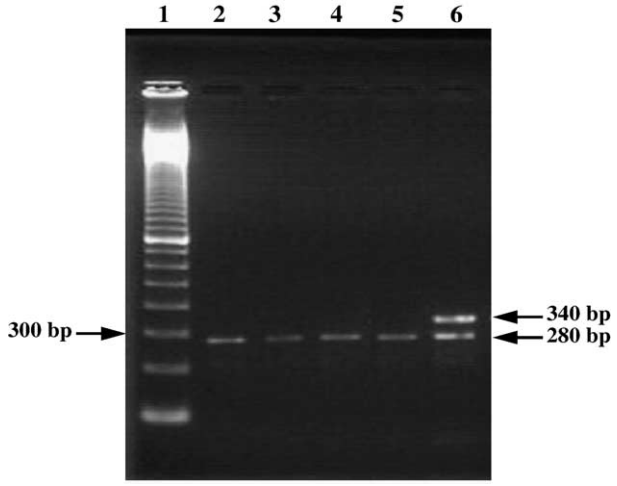


Fig. 2. Analysis of presence of any splice variant having partial intron 6 inclusion in rat brain and liver by RT-PCR. RT-PCR analysis using specific primers for detecting the presence of partial inclusion of intron 6 transcript in rat brain (lanes 2 and 3) and rat liver (lanes 4 and 5). Human brain autopsy sample from a subject expressing the partial inclusion of intron 6 (lane 6) was used as a positive control. This transcript could not be detected in rat brain and liver. A PCR amplified product of about 280 bp long representing the normally spliced CYP2D1 was detected in all the samples. Lane 1 represents 0.5 kb DNA ladder.

however, the catalytic activity is very low (1/1000) as compared to livers.

Using RT-PCR CYP2D4 was shown to be the most abundant CYP2D mRNA in rat brain [17] but the presence of the protein could not be detected using the specific antibody raised against P4502D4 even when 100 μg of microsomal protein was used [38].

Our earlier study has shown the occurrence of three alternate spliced forms of CYP2D in human brain but not in liver from the same individual. These clones have exon 3 deletion, partial inclusion of intron 6, or both [28]. The alternate spliced variant having the inclusion of intron 6 alone generated an open-reading frame (GenBank Accession Number AY220845) and metabolized codeine predominantly to morphine, unlike the wild-type CYP2D6, which forms nor-codeine as major metabolite. The alternate spliced variants containing exon 3 deletion have a premature stop codon, which prevents their translation into functional gene products. Thus, estimation of P450 isoforms by examining gene expression using Northern blotting, RT-PCR, and in situ hybridization (6) would represent contributions from functional and nonfunctional genes and would potentially overestimate the expression of a particular isoform.

In view of the important role that P4502D plays in the metabolism of psychoactive drugs, and the inability to detect the expression of P4502D4 in rat brain by earlier studies and also recent report of splice variants for CYP2D6 in human brain prompted us to investigated the presence of splice variant forms of CYP2D in rat brain using RT-PCR. Further, we have also analyzed the expression and localization of P4502D1 in rat brain using fluorescence in situ hybridization and immunohistochemistry and to assess the differences observed in the mRNA expression and the protein content in rat brain.

2. Materials and methods

2.1. Materials

cDNA to CYP2D1 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 Roche Biochemicals, 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, Bangalore, India. Animals had access to pelleted diet and

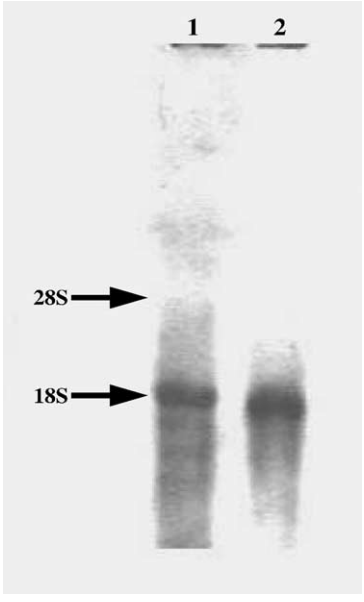


Fig. 3. Northern blot analysis of rat brain total RNA using cDNA to CYP2D1. Total RNA from rat liver (lane 1, 5 μg) and rat brain cortex (lane 2, 12 μg) were electrophoresed under denaturing conditions. After transfer to nylon membrane, the blots were hybridized with antisense Riboprobe prepared using cDNA to CYP2D1. The mobility of the 18 S and 28 S ribosomal RNA is indicated. The CYP2D1 mRNA in rat brain was seen as a band at approximately 1.6 kb.

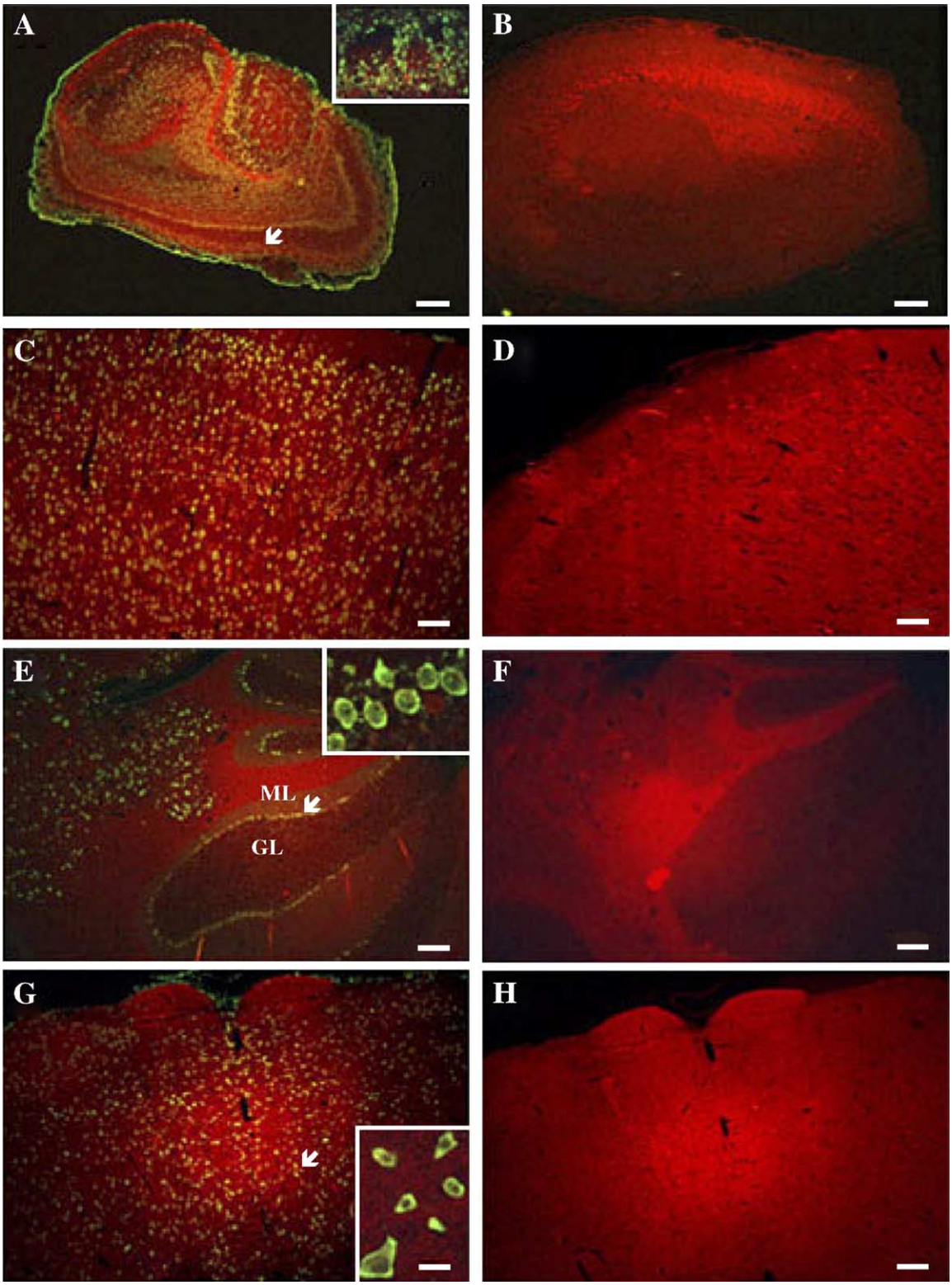


Fig. 4. Localization of CYP2D mRNA in control rat brain using fluorescent in situ hybridization. (A) In situ hybridization of coronal sections from rat brain showing intense labeling of neurons in olfactory bulb. Scale bar = 200 μm. The arrow shows the presence of CYP2D mRNA in the neurons of the glomeruli, which are depicted in inset. Scale bar = 25 μm. (B) No fluorescence staining was seen in control sections hybridized with sense probe. Scale bar = 200 μm. (C) The presence of CYP2D mRNA in the neurons of cerebral cortex clearly shows differential staining in the laminar architecture of the cortex. Scale bar = 100 μm. (D) Control section hybridized with the sense probe did not show any staining. Scale bar = 100 μm. (E) Fluorescent labeling of Purkinje neurons (arrow), granule cell layer (GL) and molecular layer (ML) of cerebellum. Scale bar = 100 μm. Inset depicting Purkinje neuron staining. Scale bar = 25 μm. (F) Control section of rat cerebellum hybridized with the sense probe and counterstained with Evan's blue is depicted. Scale bar = 200 μm. (G) The reticular neurons (arrow) in the midbrain expressed the CYP2D mRNA. Scale bar = 100 μm. Inset shows the giant reticular neuron. Scale bar = 25 μm. (H) Sections hybridized with sense Riboprobe showed no staining. Scale bar = 100 μm.

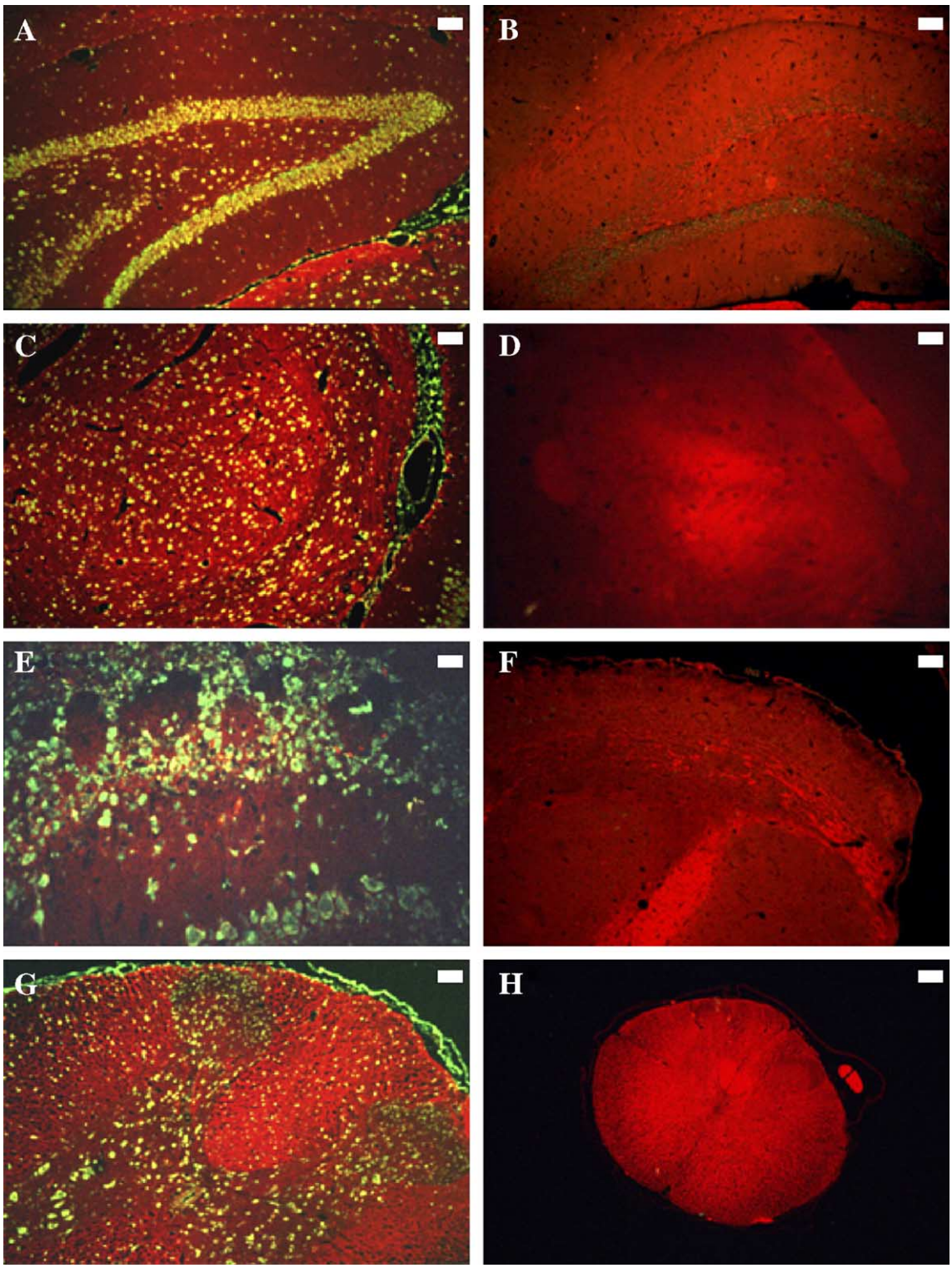


Fig. 5. Localization of CYP2D mRNA in control rat brain using fluorescent in situ hybridization. (A) The granule cell layer of the dentate gyrus (arrow) was intensely fluorescent. Intense fluorescence was seen in the CA1 pyramidal cell layer of hippocampus (double arrow). Arrowhead depicts the staining of CA3 neurons. (B) Control section hybridized with sense riboprobe showed no fluorescence. (C) CYP2D mRNA was observed in the thalamic neurons. (D) The control section hybridized with sense probe did not reveal any fluorescent staining. (E) Expression of CYP2D mRNA was seen in the neurons of the striatum although to the lesser extent than the neurons of the cerebral cortex. (F) Control sections did not show any staining. (G) The anterior horn cells of the spinal cord (arrow) were intensely fluorescent indicating the substantial expression of CYP2D mRNA. Inset depicting anterior horn cells. Scale bar = 25 μm. The position of the central canal is indicated by arrowhead. (H) The control section hybridized with CYP2D sense riboprobe did not show any fluorescent staining. Scale bars = 200 μm.

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. Studies with human brain

Human brain tissue from a traffic accident victim with no known neuro-psychiatric disorders was obtained from the Human Brain Tissue Repository, Department of Neuropathology, NIMHANS. Autopsy brain sample from a 55-year-old male with postmortem delay of 6 h was used. After autopsy, the brain was washed in ice-cold saline, the frontal cortex was dissected out based on standard anatomical markings, and stored at −70 °C.

2.4. RT-PCR analysis to identify the presence of additional transcript in rat brain and liver

Total RNA was isolated from whole brain and liver of male rats. The first strand of the cDNA was synthesized using 1 μg of total RNA from the rat brain and liver, 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. A region of the CYP2D1 cDNA representing exons 2–4 was amplified using the forward primer 5′-GTGACGTGTTCAGCCTGCA-3′ (nucleotide location 205–223 from ATG of AB008422) and the reverse primer 5′-TGAACAAAGCCGTTGTGTAA-3′ (nucleotide locations 543–562 relative to ATG of AB008422). The reaction mixture was initially denatured for 5 min at 95 °C followed by 30 cycles of denaturing for 30 s at 94 °C, annealing for 30 s at 60 °C and extension for 1 min at 72 °C. The final extension was performed for 5 min at 72 °C. Another region of the CYP2D1 cDNA representing exons 6–7 was amplified using the forward primer 5′-GGCCAAGGGGAACCCTGAGA-3′ (nucleotide location 852–871 relative to ATG of AB008422) and reverse primer 5′-GTCATACCCAGGGGGACGA-3′ (nucleotide location 1171–1189 relative to ATG of AB008422). The reaction mixture was initially denatured for 3 min at 98 °C followed by 35 cycles of denaturing for 30 s at 94 °C, annealing for 20 s at 61.2 °C and extension for 45 s at 72 °C. The final extension was performed for 7 min at 72 °C. The PCR products (20 μl) were separated by electrophoresis using 1.2% (w/v) agarose gel and stained with ethidium bromide. The identity of the PCR products was confirmed by sequencing.

2.5. Preparation of microsomes

Animals were anaesthetized with ether and perfused transcardially with ice-cold Tris buffer (100 mM, pH 7.4) containing KCl (1.15%, w/v) prior to decapitation and removal of brain. Microsomes were prepared from rat brain regions, which were dissected out using standard anatomical landmarks. Brain regions were pooled from 8–10 rats for each experiment. Tissues were homogenized using a Potter-Elvehjem homogenizer in 9 volumes of ice-cold Tris buffer (0.1 mM), EDTA (0.1 mM), KCl (1.15%, w/v), phenyl methyl sulfonyl fluoride (0.1 mM), butylated hydroxytoluene (22 μM), glycerol (20%, v/v), aprotinin (0.001%, w/v), and leupeptin (0.001%, w/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 to get the microsomal pellet. The pellet was suspended in a small volume of buffer A, aliquoted, flash-frozen in liquid nitrogen and stored at −70 °C. The protein concentration was measured by dye-binding method [4].

2.6. Immunoblot analyses

Microsomal proteins from rat brain regions (50 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis [18]. Proteins were transferred from the gel to nitrocellulose membranes [31]. Membranes were immunostained with antiserum to liver P4502D1, followed by incubation with anti rabbit IgG labeled with alkaline phosphatase (Vector Laboratories, USA). The immunostained bands were detected using nitroblue tetrazolium and 5-bromo 4-chloro 3-indolyl phosphate as chromogens.

2.7. Northern analysis and fluorescence in situ hybridization

cDNA to rat liver CYP2D1 obtained as a gift from Dr. J. P. Hardwick [10] was used for the preparation of riboprobes. The total RNA was extracted from rat brain (cortex) and liver as described by Chomezynski [7], separated electrophoretically and transferred onto positively charged nylon membrane by capillary transfer. The membranes were UV cross-linked and hybridized with digoxigenin-labeled anti-

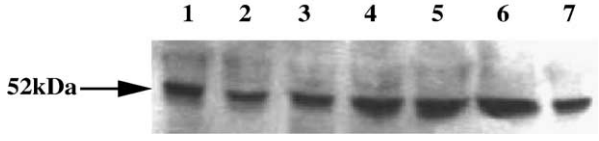


Fig. 6. Immunoblot analysis of microsomal protein from rat brain regions immunostained with antibody to rat liver P4502D1. Microsomes prepared from the rat brain regions were subjected to SDS-PAGE followed by immunoblotting. Lanes were loaded as rat liver (lane 1), rat brain cortex (lane 2), cerebellum (lane 3), brainstem (lane 4), hippocampus (lane 5), thalamus (lane 6), and striatum (lane 7). Lanes were loaded with 50 μg of microsomal protein except lane 1 which contained 10 μg of the liver microsomal protein.

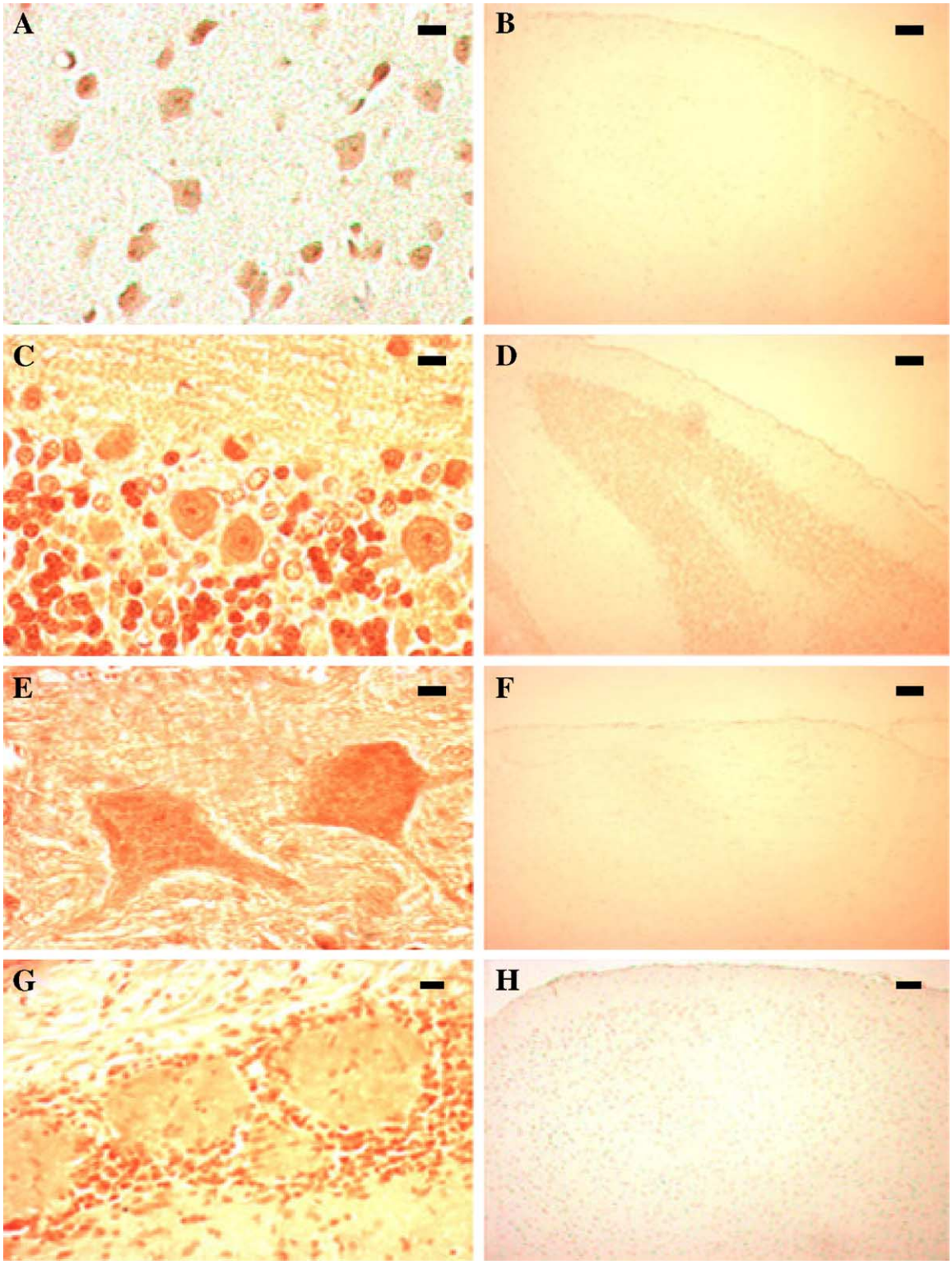
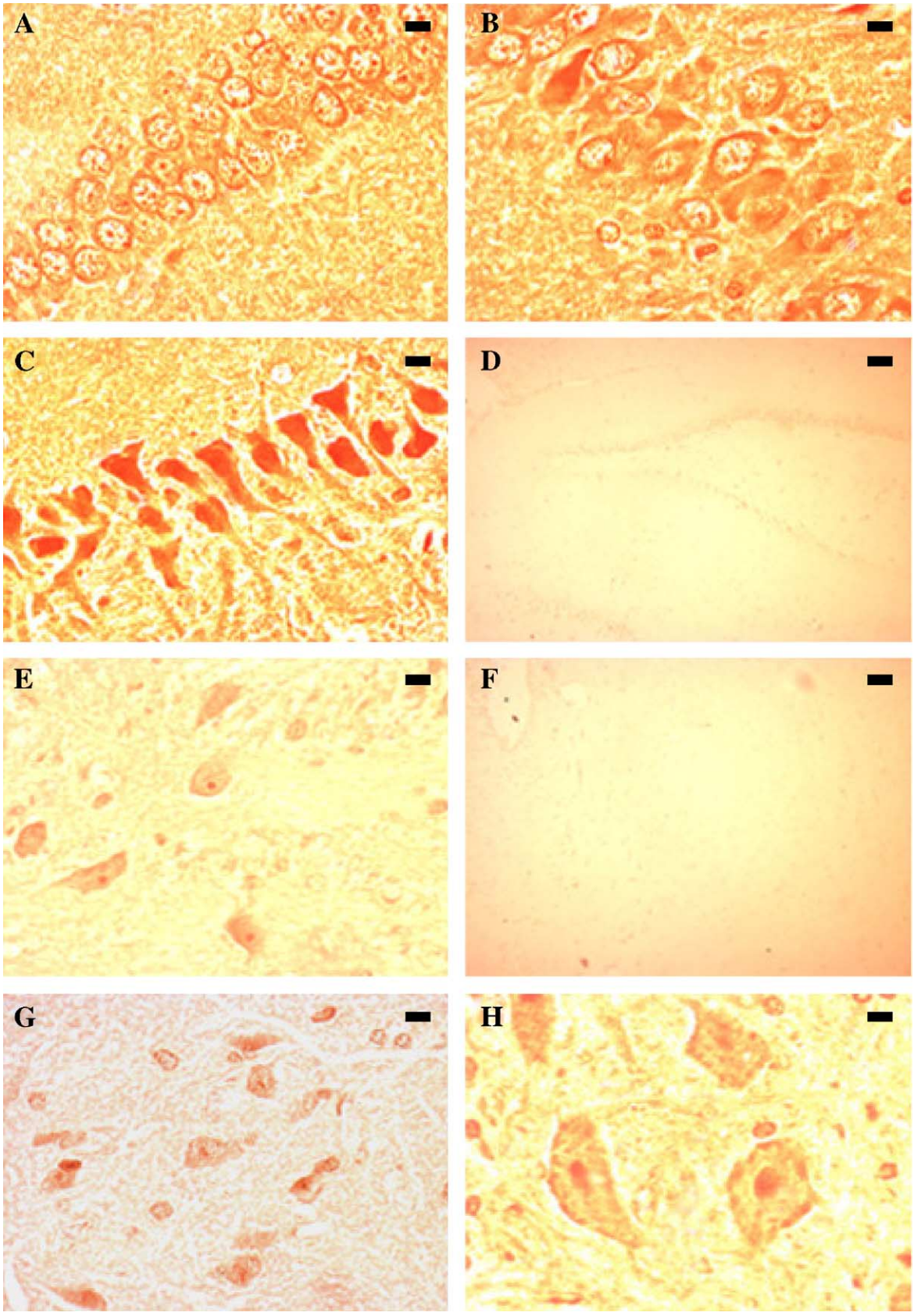


Fig. 7. Localization of P4502D1 in normal rat brain by immunohistochemistry. (A) Immunostaining of cortical neurons in the rat brain section showing the presence of P4502D1 protein. Scale bar = 10 μm. (C) Intense immunostaining of the granule cell layer of cerebellum was seen indicating the presence of P4502D1. Scale bar = 10 μm. (E) Intense immunostaining of reticular neurons in the midbrain showing the presence of P4502D1 in rat brain. Scale bar = 10 μm. (G) The neurons in olfactory glomeruli were also specifically stained demonstrating the localization of P4502D1 protein. Scale bar = 10 μm. (B, D, F, and H, respectively) Control sections of the above regions immunostained with non-immune serum did not show any immunostaining. Scale bar = 100 μm.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sense riboprobe prepared using T3 polymerase to CYP2D1. The sense cRNA probe was synthesized using T7 polymerase. The membrane was hybridized overnight with digoxigenin-labeled sense and antisense riboprobe at 55 °C, washed, incubated with antibody to digoxigenin fab fragments conjugated with alkaline phosphatase. The bands were visualized using chromogenic substrate for alkaline phosphatase.

Male Wistar rats were anaesthetized and perfused transcardially with normal saline followed by buffered paraformaldehyde (4%, w/v; 200 ml/rat) prior to the removal of brain. The tissue was processed for paraffin embedding and 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. The sections were then rinsed in phosphate-buffered saline and dehydrated using graded ethanol. Digoxigenin-labeled sense (for control sections) and antisense cRNA probes were synthesized from CYP2D1 cDNA using T3 and T7 RNA polymerases, respectively. Sections were hybridized overnight at 55 °C with sense or antisense probes. After hybridization, the sections were washed, incubated with blocking reagent (0.5% w/v, NEN Life Sciences, USA) and incubated with antibody to digoxigenin conjugated to horseradish peroxidase. After washing, the sections were incubated with biotinylated tyramide followed by streptavidin fluorescein. Finally, the sections were washed, dried, counterstained with PBS containing Evan's Blue (0.01% v/v), and mounted prior to examination under the fluorescence microscope.

2.8. Immunohistochemistry

Paraffin embedded sections of rat brains were used. Sections were dewaxed and transferred to phosphate-buffered saline containing hydrogen peroxide (3% v/v) to block the endogenous peroxidase reaction. The sections were pressure cooked in sodium citrate buffer (0.01 M, pH 6) for antigen retrieval. The sections were blocked with normal goat serum and incubated with antiserum to hepatic P4502D1 (diluted 1:1000 in PBS). Control sections were incubated with non-immune serum. The sections were washed, treated with biotinylated anti-rabbit IgG (diluted 1:1500 in PBS), and incubated with VECTASTAIN-Elite ABC reagent. The color was developed using diaminobenzidine and hydrogen peroxide. Sections were washed with water, dehydrated in graded alcohol, cleared with xylene, air dried, and mounted using Permont before examination under the microscope.

3. Results

3.1. RT-PCR analysis for detection of splice variants of CYP2D1 in rat liver and brain

RT-PCR experiments were performed to examine the presence of splice variants in the region exons 2 to 4 using cDNA prepared from rat brain and liver. The anticipated PCR product was 350 bp long representing 195–549 bp of CYP2D1. In rat brain, we observed the formation of 2 PCR products, a 350-bp band representing the normally spliced CYP2D1 and a 200-bp product representing the exon 3 deleted transcript of CYP2D1 (Fig. 1). This additional band at 200 bp was not observed in rat liver, indicating that the transcript with exon 3 deletion is not present in rat liver. We also performed RT-PCR to detect the partial inclusion of intron 6 analogous to that seen in human brain. We observed only one band at 282 bp representing the normal CYP2D1 amplicon in both rat liver and brain. As a positive control, we simultaneously RT-PCR amplified the cDNA synthesized from human brain cortex. Two bands of 340 bp and 282 bp were observed in human brain cDNA indicating the presence of the brain variant CYP2D containing partial inclusion of intron 6 (Fig. 2).

3.2. Constitutive expression of P4502D in rat brain

The constitutive expression of CYP2D in the rat brain was examined by Northern blot analysis performed with total RNA from rat liver and brain cortex using the riboprobe synthesized from the cDNA to CYP2D1. Northern blot analysis using the T3 RNA riboprobe (antisense) revealed the constitutive expression of CYP2D mRNA in rat brain. The molecular mass of the transcript was approximately 1.6 kb (Fig. 3). No signal was observed when Northern blots were hybridized with the sense riboprobe, which was synthesized from cDNA to CYP2D1 using T7 polymerase (data not shown).

3.3. Localization of CYP2D mRNA in rat brain by fluorescence in situ hybridization

Fluorescence in situ hybridization experiments were performed using normal rat brain sections to determine the localization of CYP2D mRNA. Fluorescence in situ hybridization studies demonstrated the presence of CYP2D mRNA predominantly in neuronal cells in rat brain regions, which

Fig. 8. Localization of P4502D1 in normal rat brain by immunohistochemistry. (A) Intense immunostaining indicating the presence of P4502D in CA1 pyramidal neurons of hippocampus. Scale bar = 10 μm. (B) Positive staining of P4502D protein was observed in the CA2 neurons of hippocampus. Scale bar = 10 μm. (C) CA3 neurons of hippocampus also showing the presence of P4502D1 protein. Scale bar = 10 μm. (D) Control section of hippocampus stained with non-immune serum did not show any immunostaining for P4502D1 protein. Scale bar = 200 μm. (E) Presence of P4502D1 protein was seen in the neurons of thalamus. Scale bar = 10 μm. (F) Immunolabeling was not observed in the control section of thalamus treated with non-immune serum. Scale bar = 200 μm. (G) Sparsely stained striatal neurons showing the presence of P4502D1 protein in rat brain. Scale bar = 10 μm. (H) Staining was observed in anterior horn cells of spinal cord section incubated with P4502D1 antiserum. Scale bar = 10 μm.

were hybridized with the riboprobe synthesized from the cDNA to CYP2D1. A high level of CYP2D1 mRNA expression was seen in olfactory bulb, thalamus, cerebral cortex, and hippocampus. In the olfactory lobe, the neuronal cells and glomeruli (inset) were intensely labeled (Fig. 4A), whereas control section did not show any staining when hybridized with sense probe (Fig. 4B). The neurons in the cerebral cortex showed intense cytosolic staining, indicating the presence of CYP2D mRNA (Fig. 4C), while the section hybridized with sense probe did not show any fluorescence (Fig. 4D). The laminar architecture of different cortical layers was clearly seen in the section hybridized with antisense probe. In the cerebellum, Purkinje cell layer showed intense fluorescence, while the interneurons of the molecular layer (ML) were relatively less stained (Fig. 4E). Higher magnification of Purkinje neurons showed intense fluorescence in the cell body of the neurons (inset). In the midbrain, the reticular neurons were selectively labeled, indicating the predominant presence of CYP2D mRNA within these cell population (Fig. 4G). Intense fluorescence was seen in hippocampus, in the pyramidal neurons of CA1, CA2, and CA3, and in the granule cell layer in the dentate gyrus (Fig. 5A). The thalamic neurons are also stained intensely showing the expression of CYP2D mRNA (Fig. 5C), whereas control section hybridized with sense probe did not show any staining (Fig. 5D). There was only sparse staining of neurons in the striatum (Fig. 5E). The anterior horn cells of the spinal cord (inset) are intensely fluorescent indicating the expression of CYP2D mRNA (Fig. 5G), while the control section hybridized with sense probe did not show any staining (Fig. 5H).

3.4. Immunoblot analysis of microsomes from rat brain using antiserum to hepatic P4502D

Immunoblot studies were carried out to confirm the presence of the P4502D in various regions of rat brain. Immunoblot analysis of microsomes prepared from rat brain regions using antiserum to the hepatic P4502D1 revealed the presence of an immunoreactive protein of molecular weight 52 kDa. The constitutive presence of P4502D was detectable in all the rat brain regions examined. There was a variation in the expression of P4502D among the regions studied, more staining was seen in brainstem, hippocampus, and thalamus and comparatively less intense staining was observed in striatum (Fig. 6).

3.5. Immunohistochemical localization of P4502D1 in rat brain

Immunohistochemical studies demonstrated the presence of P4502D1 protein predominantly in neuronal cells in rat brain. Higher magnification of cortical neurons in rat brain expressing P4502D1 is shown in Fig. 7A. Immunostaining of apical dendrites was also observed. No immunostaining was seen in sections pretreated with non-immune serum (Fig. 7B). Intense immunostaining was observed in neurons of olfactory bulb, indicating the presence of P4502D1. The neurons in the olfactory glomeruli were also intensely stained (Fig. 7G). No immunostaining was observed in control sections incubated with non-immune serum (Fig. 7H). Presence of P4502D1 protein was observed in CA1, CA2, and CA3 subfields of hippocampus (Figs. 8A, B, and C). P4502D1 expression was also seen in the neurons of the anteroventral nucleus of thalamus (Fig. 8E). Sparse staining of neurons was observed in striatum indicating the presence of P4502D1 protein (Fig. 8G). Intense staining of anterior horn cells in spinal cord demonstrated the constitutive expression of P4502D1 in these cells (Fig. 8H).

4. Discussion

Several P450 isoforms, such as CYP2D, are present in rat brain and localize predominantly in neurons, the site of action of most drugs [15,17,26,38]. However, the mRNA expression does not correlate with the protein levels. The presence of unique, tissue-specific isoforms of P450 generated through alternate splicing provides a mechanism by which a variety of transcripts are generated. However, premature termination of translation of these transcripts would results in the absence of the functional protein.

Polymorphisms of CYP2D1 for rat and CYP2D6 for humans have been associated with impaired oxidation of many drugs with diverse pharmacological actions [20]. Rat CYP2D1 and human CYP2D6 gene shares 83% homology. There is some indication that CYP2D6 mRNA splice variants are produced in human liver [10,11]. Studies from our laboratory have shown the presence of the splice variant forms of CYP2D in human brain [28]. In the present study, we used RT-PCR to identify the presence of any such splice variants of CYP2D1 in rat brain and liver (Fig. 1) and discovered the presence of a splice variant with exon 3 deletion in the rat brain but not in liver. The deletion of exon 3 results in the generation of a stop codon at 391–393 bp resulting in premature termination of translation (Fig. 1B). Thus, the mRNA detected does not translate into a functional protein.

Nervous system has a propensity for generating alternate spliced forms and splicing defects cannot be related to differences in the genomic sequence but may be regulated by mechanisms involving spliceosomal complex and RNA binding proteins, which are poorly understood [12]. An alternate spliced form of flavin-containing monooxygenase (FMO4) with exon 4 deletion is seen in rat brain but not in other tissues [16]. The tendency for rat brain to generate alternate spliced genes is seen in the present study wherein an alternate spliced variant having exon 3 deletion was identified only in the rat brain and not in liver. Screening of a human cDNA library revealed the presence of three variants namely, exon 3 deletion,

partial inclusion of intron 6 and a third having both the deletion and inclusion [28]. However, the splice variant with partial inclusion of intron 6 detected in human brain was not observed in rats while the variant with exon 3 deletion was detected (Fig. 2).

The alternate spliced variants containing exon 3 deletion have a premature stop codon, which prevents their translation into functional gene products. Thus, estimation of P450 isoforms by studying gene expression alone using Northern blotting, RT-PCR, and in situ hybridization [12,16,19] would represent contributions from functional and prematurely terminated genes and would potentially over-estimate the expression of a particular isoform. This could explain the high levels of expression CYP2D1 mRNA seen in the present study, but not the corresponding protein. High levels of expression of a P450 mRNA with no accumulation of the corresponding protein have been convincingly demonstrated in the case of P4502C13 in the livers of rats [22]. This study also demonstrates that within the same species there exists a difference in expression of P450 alternate spliced forms in brain and liver.

In conclusion, the study demonstrates the significant differences that exist in the presence of alternate spliced forms in liver and brain. The presence of as yet unidentified P450 forms generated by alternate splicing would help understand the specific biotransformation pathways occurring at the target site of action of drug(s). It further indicates that CYP2D1 mRNA is widely and constitutively expressed in neuronal cells in the rat brain. Identification of new isoforms of P450 at target site needs an in depth analysis of at the mRNA and protein level. Several hepatic forms, such as, CYP1A2, 2B1, 2B2, 3A1 [30], 2C6, 2C11, 2C12, 2C23, and 2E1 [39], have been detected by PCR, but the corresponding proteins may contribute very little to the overall content of brain P450.

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