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Cytochromes P450 of the 2D Subfamily in Rat Brain

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SUMMARY

The possible relationship between cytochrome P450 (P450) in brain and degenerative diseases of the central nervous system prompted an investigation into the members of the 2D subfamily in rat brain. The mRNA for P450 2D4 was much more abundant than those for 2D1 and 2D5, which are major hepatic forms. 2D2 and 2D3 mRNAs, which are also abundant forms in liver, were not detectable in brain. To evaluate the quantitative significance of 2D4 protein in brain, specific antibodies were raised. The full length 2D4 cDNA was cloned from brain mRNA by reverse transcription-polymerase chain reaction amplification and was translated, in a reticulocyte lysate system, into a protein of approximately 50 kDa. A [^{35}S]methionine-labeled protein of 50 kDa could be immunoprecipitated from *in vitro* translated 2D4 mRNA. In Western blots, no signals were obtained with brain microsomes. However, with P450 extracted from brain a band of 50 kDa could be detected when 60 pmol or more were loaded in each lane. There was no detectable developmental regulation of the 2D4 mRNA and no change during pregnancy, during lactation, or after treatment with ethanol, conditions under which the P450 content of brain increases. In Dark Agouti rats, which do not express P450 2D1 mRNA in liver, 2D4 mRNA in brain was as abundant as in Wistar rats. We conclude that 2D4 is expressed as a stable protein in the brains of untreated rats, where it represents <5% of the total P450.

P450s are a large superfamily of mixed-function oxidases that are found in all organisms from bacteria to humans. They have been classified into families and subfamilies on the basis of sequence homologies. Enzymes of several families are important in the biosynthesis of endogenous components, including steroids, cholesterol, bile acids, and fatty acid metabolites, whereas members of other families and subfamilies are mainly involved in the metabolism of drugs, food components, and environmental contaminants (for review, see Refs. 1 and 2). The P450 2D subfamily has evolved differently in humans and rats. In rats five genes have been described (*2D1–5*), whereas in humans one gene (*2D6*) and several pseudo-genes (*2D7P* and *2D8P*) are known (2). For both species polymorphisms have been described. In the human population 5–10% of Caucasians exhibit defects in *2D6* alleles, with resultant decreased rates of metabolism of 2D6 substrates (3, 4); Sprague-Dawley rats have a variant *2D1* allele, *2D1v*, whereas Dark Agouti rats have no detectable expression of 2D1 mRNA in the liver (5).

Human 2D6 and rat 2D1 have been shown to metabolize a variety of drugs, among them β-adrenergic receptor blockers, analgesics, and psychoactive compounds such as neuroleptics, tricyclic antidepressants, and monoamine oxidase inhibitors (for review, see Ref. 6). One of the characteristic substrates for 2D1 and 2D6 is the β-adrenergic receptor blocker bufuralol. No bufuralol-1-hydroxylase activity could be measured when the cDNAs for 2D2, 2D3, and 2D5 were expressed in a heterologous expression system. 2D1v had <10% of the 2D1 activity (7). 2D4 was not tested, because complete cDNA was not available.

There are several epidemiological studies investigating whether defects in *2D6* alleles are associated with an increased risk for the development of Parkinson's disease. Some studies show an over-representation of poor metabolizers among patients with Parkinson's disease (8, 9) and some do not (10). In one study (11), a specific mutation in the *2D6* coding region was closely associated with Parkinson's disease. Interest in the relationship between P450 and Parkinson's disease arises from the hypothesis that Parkinson's disease is caused by some endogenous or environmental neurotoxins that are normally detoxified by 2D6. When the liver enzyme is defective, neurotoxins accumulate and neurodegeneration results. One such naturally occurring compound, tetrahydroisoquinolone, which causes parkinsonism when administered to monkeys (12), is metabolized by 2D P450s (13). Because in Parkinson's disease there is degeneration of

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ABBREVIATIONS: P450, cytochrome P450; RT, reverse transcription; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; SSC, standard sodium citrate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPOA, hypothalamic preoptic area; PBS, phosphate-buffered saline.

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neurons, it is pertinent to ask whether the 2D P450s in brain are of relevance for the *in situ* metabolism and inactivation of neurotoxins. Although 2D6 and 2D1 mRNAs are mainly expressed in the liver, 2D6 mRNA has been detected in human brain (14), and catalytic activity corresponding to 2D P450 has been detected in canine (14), rat (15), rabbit, and bovine brain (16). These catalytic activities are very low (1/1000 of the liver levels). Because there are at least five distinct 2D P450s in rats, it is necessary to determine which of these are expressed in the central nervous system. Recently, Komori (17) has shown with that the most abundant 2D mRNA in brain is that of 2D4. Several other hepatic forms, i.e., 1A2, 2B1, 2B2, 3A1 (18), 2C6, 2C11, 2C12, 2C23, and 2E1 (19), have been detected in brain by PCR, but the corresponding proteins contribute very little to the overall content of brain P450 and are not detectable on Western blots with brain microsomes or P450 extracted from brain (for review, see Ref. 20). Because almost 90% of the P450 remains to be identified and because the 2D4 mRNA appeared to be relatively abundant, this study was undertaken to identify and quantitate the 2D4 protein in rat brain.

Materials and Methods

Animals. Wistar, Dark Agouti (180–200 g), and timed pregnant rats were obtained from Møllegaard (Ejby, Denmark). They were kept under controlled conditions of temperature and humidity and had free access to food and water. For ethanol induction studies a single dose of ethanol (200 μl) was injected intraperitoneally. Rats were killed by decapitation after CO_2 narcosis, 3, 6, or 16 hr after ethanol treatment. Heads were cooled on ice before dissection of the brains.

Enzymes and reagents. Restriction enzymes, *Thermus aquaticus* polymerase, avian myeloblastosis virus reverse transcriptase, SP6 polymerase, the RNase inhibitor RNasin, RNase-free DNase, Sephadex G-50 nick columns, the reticulocyte lysate transcription/translation system, pGEM7Zf+, deoxynucleotide triphosphates, and oligo(dT) were purchased from Promega (Madison, WI); radionucleotides, L-[^{35}S]methionine, and enhanced chemiluminescence Western blot reagents from Amersham (Buckinghamshire, England); PMSF, BSA, salmon sperm DNA, and goat anti-rabbit IgG-peroxidase conjugate from Sigma Chemical Co. (St. Louis, MO); tissue cell culture media and L-glutamine from Life Technologies (Paisly, Scotland); GeneScreen Plus membranes from DuPont-NEN (Boston, MA); *N*-succinimidyl-3-(2-pyridyldithiopropionate) from Pierce (Rockford, IL); lysine-Sepharose 4B, Protein A-Sepharose CL-4B, Ficoll (type 400), and pSVL from Pharmacia (Uppsala, Sweden); Emulgen from Kao Atlas (Tokyo, Japan); Nonidet P-40 and Tween-20 from Kebo Lab AB (Stockholm, Sweden); Pro Blott membranes from Applied Biosystems (Foster City, CA); 4-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate from Boehringer (Mannheim, Germany); and goat anti-rabbit IgG(H+L)-alkaline phosphatase conjugate and gelatin from Bio-Rad (Richmond, CA). All standard reagents were supplied by Merck (Darmstadt, Germany). Unless otherwise indicated, the pH values of the Tris·HCl buffers were adjusted at room temperature.

Isolation of total RNA. Total RNA was isolated by the guanidinium thiocyanate single-step method described by Chomczynski and Sacchi (21).

RT. Total RNA (5 μg) derived from various tissues was added to 10 mM Tris·HCl, pH 9, 50 mM KCl, 7.5 mM $MgCl_2$, 0.1% Triton X-100, with 1 mM levels of each deoxynucleotide triphosphate, 2 μM levels of conserved 2D antisense primer or oligo(dT) primer, and 20 units of reverse transcriptase. cDNA synthesis was carried out in a total volume of 50 μl for 45 min at 42° and was terminated by heating to 95° for 10 min. Reactions were then chilled on ice.

PCR amplification. A 2-μl aliquot of each RT reaction was used for PCR amplification with either the conserved 2D or the 2D4-specific PCR primer pair. PCR was performed for 30 cycles in a thermal cycler from Perkin-Elmer/Cetus (Norwalk, CT). The conditions were 94° for 30 sec, 57° for 30 sec, and 72° for 60 sec. The reaction was carried out in 50 mM KCl, 10 mM Tris·HCl, pH 9, 0.1% Triton X-100, 2.5 mM $MgCl_2$, with 0.2 mM levels of each deoxynucleotide triphosphate, 1 μM levels of each PCR primer, and 20 units of *T. aquaticus* polymerase, in a total volume of 100 μl.

PCR amplification of 2D sequences. 2D sense (5′-GCAGCGAAGCTTACCAATGCTGTCATCCATGAGGT-3′) and antisense (5′-ATCTATGGATCCGGCATGAAGGCCTCATGCTTCAC-3′) primers used for amplification of 2D cDNA are identical to conserved 2D regions of the sense strand (nucleotides 1075–1097) and the antisense strand (nucleotides 1291–1313), respectively. For cloning purposes, the sense primer was designed with a *Hind*III restriction site (5′-GCAGCGAAGCTT-3′) and the antisense primer with a *Bam*HI restriction enzyme site (5′-ATCTATGGATCC-3′).

Southern blot analysis of PCR products. PCR products obtained by using the conserved 2D primers were separated on 2% agarose gels. Duplicate 6-μl aliquots of the PCR products were analyzed. DNA was transferred to GeneScreen Plus membranes by capillary action, in 0.4 M NaOH, for 16 hr. Membranes were air-dried and baked for 1 hr at 80° before prehybridization in 6× SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7), 10× Denhardt's solution [0.2%, w/v, Ficoll (type 400), 0.2%, w/v, polyvinylpyrrolidone, 0.2%, w/v, BSA (fraction V)], 0.01 mM EDTA, 1% SDS, 200 μg/ml denatured salmon sperm DNA, for 4 hr at 65°. Hybridization was performed in 10 ml of 6× SSC, 10× Denhardt's solution, 0.01 mM EDTA, 1% SDS, with about 10×10^6 cpm of labeled oligonucleotides specific for either 2D1, 2D2, 2D3, or 2D4, at 42° for 16–18 hr. Before autoradiography, filters were washed for 3 × 10 min in 6× SSC at a temperature of 3° below the calculated melting temperature.

Oligonucleotide probe preparation. The following probes were used: 2D1/5, 5′-GCCACGCATCACGAGTTGT-3′ (coding for nucleotides 1131–1149); 2D2, 5′-ACCACATATGACATCCCGA-3′ (coding for nucleotides 1131–1149); 2D4, 5′-GCCTCACAAGACTTCTCGT-3′ (coding for nucleotides 1131–1149); 2D3, 5′-GTCCCAATGAATTTGCCAC-3′ (coding for nucleotides 1117–1135). Oligonucleotides were end-labeled with T4 polynucleotide kinase (22), purified over nick columns, and diluted with nonlabeled oligonucleotides to similar specific activities.

Northern blot analysis. RNA aliquots of 20 μg were subjected to denaturing electrophoresis on agarose-formaldehyde gels and transferred to nylon membranes as described previously (23, 24).

2D4 riboprobe preparation. 2D4-specific sense (5′-ATCATGAAGCTTAGACTTCCAGAATATGCCAGCGG-3′) and antisense (5′-TCTCACGGATCCCAAAGCCCGACTGGTCATTGAAA-3′) primers designed with a *Hind*III (5′-ATCATGAAGCTT-3′) and a *Bam*HI (5′-TCTCACGGATCC-3′) restriction site, respectively, were used for RT-PCR of total brain RNA. The sense primer corresponds to nucleotides 156–178 of the 2D4 coding region, whereas the antisense primer is complementary to the sequence between nucleotides 324 and 346.

The PCR product was inserted into the *Hind*III/*Bam*HI restriction sites of pGEM7Zf+ and verified by sequencing. To synthesize a riboprobe complementary to the 2D4 sense strand, *Hind*III-linearized plasmid was used as a template for SP6 RNA polymerase. The reaction was performed with 1 μg of plasmid DNA in 40 mM Tris·HCl, pH 7.5, with 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 100 μCi of [α-^{32}P]UTP (800 Ci/mmol), 40 units of the RNase inhibitor RNAsin, and 20 units of SP6 RNA polymerase, in a total volume of 20 μl, for 1 hr at 37°. DNA was removed by incubation with 1 unit of RNase-free DNase at 37° for 15 min. Excess [α-^{32}P]UTP was removed by purification of the cRNA on a nick column. The riboprobe was immediately used for hybridization.

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Hybridization with 2D4 riboprobe. Membranes were prehybridyzed in 6× SSC, 1% BSA, 1% SDS, 50% formamide, 200 μg/ml salmon sperm DNA, for 2–4 hr at 68°. Hybridization was carried out at the same temperature for 16–18 hr, in 10 ml of prehybridization solution containing 15–30 × 10^6 cpm of [α-^{32}P]UTP-labeled 2D4 riboprobe. Filters were washed three times for 5 min with 2× SSC/1% SDS, washed twice for 5 min and once for 90 min with 0.1× SSC/1% (w/v) SDS at 68°, and exposed to Kodak X-Omat AR film at −70°, using intensifying screens.

Actin DNA probe preparation. A PCR-derived actin DNA fragment corresponding to nucleotides 211–727 of the chicken actin coding region was purified on a nick column and labeled with [α-^{32}P]dCTP (3000 Ci/mmol), using the Megaprime DNA labeling kit from Amersham.

Hybridization with actin probe. Membranes were prehybridized in 6× SSC, 1% (w/v) BSA, 1% SDS, for 4 hr at 68°. Hybridization was performed for 16–18 hr at 68°, in 10 ml of prehybridization solution containing about 10–20 × 10^6 cpm of labeled actin probe. After hybridization, filters were washed twice for 5 min in 2× SSC/1% (w/v) SDS at 68° and three times for 40 min in 0.1× SSC/1% (w/v) SDS at room temperature.

Plasmid constructs for *in vitro* transcription/translation and COS-7 cell expression. The following primers were used for RT-PCR cloning of 2D4 and 2D1v*[1]: 2D4 sense, 5′-CTTCACCATATGCGTATGCCGACGGGTTCTGAA-3′; 2D4 antisense, 5′-TTGCTAGGATCCATTAGCGGGGTGAAGCACAGA-3′; 2D1 sense, 5′-AAGGCACATATGGAGCTGCTGAACGGCACTGGC-3′; 2D1 antisense, 5′-GAGCTGGGATCCATTAGAGTCCTTGCTCCCGTA-3′. To allow subcloning into the bacterial expression vector pSPORT19 (25), sense primers were synthesized with a *Nde*I restriction site and antisense primers with a *Bam*HI restriction site. The changes in the sense primers were introduced to facilitate expression in bacteria. Amplified products were subcloned and analyzed by DNA sequencing. pSPORT19 2D constructs were used for *in vitro* transcription and translation.

For expression in COS-7 cells, the 2D4 fragment was excised from pSPORT19 2D4 by restriction with *Eco*RV and *Bam*HI and was subcloned into the *Sma*I/*Bam*HI restriction sites of pSVL. The final pSVL 2D4 construct was obtained by excision of the *Xba*I fragment, to place the 2D4 start codon in the proximity of the late promoter.

pSVL 2D1v was constructed by transfer of the *Eco*RI 2D1v fragment from pSP64 2D1v (kindly provided by U. M. Zanger, Biocenter of the University of Basel, Basel, Switzerland) into the *Eco*RI site of pGEM7Zf+. Correctly oriented 2D1v fragment was then subcloned into the *Xba*I/*Bam*HI restriction sites of pSVL.

Peptide synthesis and immunization of rabbits. Peptide synthesis and immunization were carried out by Med Probe AS. (Oslo, Norway). The following peptides were synthesized with a carboxyl-terminal tyrosine residue to allow coupling to the carrier protein keyhole limpet hemocyanin: Pep1, HFNDQSGFGPRS (corresponding to amino acids 108–119 of 2D4 protein); Pep2, KAKGNPESSFND (corresponding to amino acids 284–295 of a conserved region within the rat 2D subfamily).

Affinity purification of anti-2D and anti-2D4 antibodies. Lysine-Sepharose 4B slurry containing about 4–5 μmol of lysine was incubated with an equimolar amount of *N*-succinimidyl-3-(2-pyridyldithiopropionate) dissolved in dimethylsulfoxide. The reaction was performed in 10 ml of 2 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl and 1 mM EDTA, at 22° for 30 min with constant rocking. Coupling efficiency was tested according to the manufacturer's instructions and was about 50–60%.

After removal of excess cross-linking agent by washing of the Sepharose matrix several times with 2 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl, 1 mM EDTA, the matrix was incubated with 4 μmol of the peptide (dissolved in 500 μl of PBS) for 18 hr at 22°. The following peptides were synthesized by Scandinavian Peptide Synthesis AB (Köping, Sweden): HFNDQSGFGPRSC for purification of anti-2D4 antibodies and EKAKGNPESSFNDEC for purification of anti-2D antibodies. To saturate free reactive groups, the matrix was then incubated with 1 ml of 100 mM cysteine for 3 hr at 22°. Cysteine was removed by extensive washing of the Sepharose matrix with 2 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl, 1 mM EDTA.

Affinity purification was performed as described (26). Affinity-bound antibodies from 1 ml of serum were eluted in 10 ml of 100 mM glycine, pH 2.5, and dialyzed against PBS.

Metabolic labeling and lysis of COS-7 cells. Cells were grown on 6-cm-diameter plates to 80% confluency, washed twice with prewarmed minimal essential medium with Earle's salts, without L-methionine and L-glutamine, and incubated for 4 hr in 2 ml of medium supplemented with 2 mM L-glutamine and 40 μCi of L-[^{35}S]methionine (>1000 Ci/mmol). After labeling, cells were washed twice with cold PBS and then lysed on the plate at 4° for 20 min, in 400 μl of 120 mM NaCl, 50 mM Tris·HCl, pH 7.5 at 4°, 5 mM EDTA, 1% (v/v) PMSF. Before incubation with antiserum, lysates were precleared by centrifugation at 10,000 × *g* for 10 min at 4°.

Immunoprecipitation. Lysates were incubated in the presence of 10 μl of rabbit anti-2D antiserum at 4° for 1 hr. After incubation, 80 μl of Protein A-Sepharose CL-4B matrix were added and lysates were further incubated for another 1 hr at 4°. The Protein A-Sepharose matrix was washed twice with 140 mM NaCl, 50 mM Tris·HCl, pH 7.5 at 4°, 5 mM EDTA, 1% (v/v) Nonidet P-40, once with the same solution without detergent, and once with water. Samples were then incubated for 5 min at 95° in SDS sample buffer before analysis by SDS-PAGE.

Preparation of brain P450. Brain P450 was prepared from a solubilized brain total membrane preparation as described previously (27). A 20% (w/v) homogenate in 0.25 M sucrose containing 1 mM EDTA and 10 mM potassium phosphate buffer, pH 7.5, was prepared with a Polytron homogenizer. PMSF (0.2 mM) was added and a total membrane preparation was sedimented by centrifugation at 100,000 × *g* for 1 hr. Membranes were resuspended in solubilization buffer composed of 50 mM potassium phosphate buffer, pH 7.5, 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, 0.2% (v/v) Emulgen 911, and 0.2 mM EDTA. PMSF was again added. After 3–16 hr, insoluble material was sedimented by centrifugation at 100,000 × *g* for 1 hr. The solubilized material was diluted 4-fold with 50 mM potassium phosphate buffer containing 20% (v/v) glycerol and was chromatographed on a column (5 × 2.5 cm) of *p*-chloroamphetamine-coupled Sepharose, as described previously (27). The column was washed with 100 ml of solubilization buffer diluted 1/4, and P450 was eluted with solubilization buffer. P450 was quantitated spectrally (28), and aliquots were taken for Western blots. The rest of the P450 was dialyzed overnight against 1 liter of 20 mM potassium phosphate buffer, pH 7.4, containing 50 mM potassium chloride and 20% (v/v) glycerol. This was followed by chromatography on a column (3.0 × 2.0 cm) of DE-52 cellulose equilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 50 mM potassium chloride, 20% glycerol, and 0.2% (v/v) Emulgen 911. Approximately 60% of the P450 was recovered in the flow-through fraction, and no P450 could be eluted from the column with increasing concentrations of potassium chloride.

Proteins were precipitated with chloroform/methanol and resolved on 10% SDS-polyacrylamide gels according to the method of Laemmli (29). Approximately 80 pmol of P450, equivalent to 1.5–5 μg of P450 protein, were applied to each lane.

Western blot analysis. After SDS-PAGE, proteins were transferred onto Pro Blott membranes in a semidry blotter, in 125 mM Tris, 960 mM glycine (1.2 mA/cm^2 of membrane for 2 hr). Blots were blocked for 2–3 hr at 30° with 3% (w/v) gelatin dissolved in 150 mM NaCl, 20 mM Tris·HCl, pH 7.5. All further incubations were at room

[1] 2D1 differs from 2D1v in four amino acids, at amino acid positions 123, 124, 173, and 380. 2D1 has valine, phenylalanine, arginine, and isoleucine, whereas 2D1v has isoleucine, leucine, glutamine, and phenylalanine in these positions. We were unable to clone 2D1 from the livers of Sprague-Dawley rats. The sequence we obtained was 2D1v except for amino acid position 380, which was isoleucine. This cDNA clone is referred to as 2D1v*.

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temperature. Incubations with primary and secondary antibodies were performed for 1–2 hr in buffers composed of 1% (w/v) gelatin, 150 mM NaCl, 20 mM Tris·HCl, pH 7.5, and 0.05% (w/v) Tween-20. The following antibody dilutions were used: rabbit anti-2D antiserum, 1/500; purified antibodies, 1/25; alkaline phosphatase-conjugated goat anti-rabbit IgG(H+L), 1/3000. After incubation with the first antibody, blots were washed twice in 150 mM NaCl, 20 mM Tris·HCl, pH 7.5, 0.05% (w/v) Tween-20, for 5 min. After incubation with secondary antibody, blots were washed twice in 150 mM NaCl, 20 mM Tris·HCl, pH 7.5, 0.05% (w/v) Tween-20, once in 150 mM NaCl, 20 mM Tris·HCl, pH 7.5, and once in 100 mM Tris·HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, for 5 min each. Bands were visualized with 4-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as described (22). When the enhanced chemiluminescence detection system was used, the secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG. Blots were blocked by an overnight incubation at 5° with 10% (w/v) fat-free milk in 150 mM NaCl, 3 mM KCl, 10 mM phosphate buffer, pH 7.4, 1% (v/v) Nonidet P-40. Incubations with primary and secondary antibodies were performed for 1 hr with 3% (w/v) fat-free milk in 150 mM NaCl, 3 mM KCl, 10 mM phosphate buffer, pH 7.4, 1% (v/v) Nonidet P-40, at 1/25 and 1/3000 dilutions, respectively. Washes were performed with blocking solution.

Results

RT-PCR analysis of brain 2D mRNAs in Dark Agouti rats. It has been shown that in the livers of Dark Agouti rats there is no detectable expression of the *2D1* gene but normal expression of the *2D5* gene (5). Expression of 2D4 mRNA has not been reported in this rat strain. We, therefore, used RT-PCR to investigate which of the 2D mRNAs are expressed in rat brain. The PCR primers chosen recognize DNA regions that are conserved within the 2D subfamily and ought to amplify cDNA sequences of all members of the rat 2D subfamily. PCR amplification was performed with reverse-transcribed total RNA derived from the HPOA, cerebellum, cortex, and olfactory bulbs. Liver cDNA was used as a positive control. PCR-amplified products were separated on agarose gels, transferred to nylon membranes, and probed with labeled oligonucleotide probes (of similar specific activities) specific for 2D1 and 2D5 (2D1/5), 2D2, 2D3, or 2D4. Before exposure, membranes were washed under stringent conditions established for each of the oligonucleotide probes. 2D1/5, 2D2, 2D3, and 2D4 sequences could be amplified to similar degrees from liver cDNA, whereas with cDNA from different brain regions there was amplification of mainly 2D4 DNA sequences (Fig. 1A). After longer exposure of the autoradiograms, signals for 2D1/5 but not 2D2 and 2D3 were evident (Fig. 1B). This result could mean either that 2D1 is expressed in brain but not in liver of Dark Agouti rats or that in this rat strain 2D5 instead of 2D1 is expressed in brain.

Identical results were obtained with RNA isolated from the brains of Wistar rats (data not shown). Because the 2D1/5 oligonucleotide recognizes both 2D1 and 2D5 sequences, we do not know whether 2D1 or 2D5 mRNA is expressed in brain in Wistar rats. 2D4 was also the main 2D sequence amplified by RT-PCR using female and newborn rat brain total RNA (data not shown).

Cloning of 2D4 cDNA. To test whether full length 2D4 mRNA is present in brain, PCR amplification was done with primers containing sequences corresponding to the 5′ and 3′ regions of the 2D4 cDNA, as deduced from the genomic sequence. PCR amplification resulted in a visible band of the correct size on an ethidium bromide-stained agarose gel. The band was isolated and used for ligation into pSPORT19 (25). The DNA of several of the subcloned fragments was sequenced. The DNA sequence of one subclone corresponded to the deduced 2D4 cDNA sequence. Other subclones contained single mutations, probably due to misincorporation of nucleotides during PCR amplification.

Northern blot analysis of 2D4 mRNA. Northern blot analysis was used to study the expression of 2D4 in various brain regions. Because the P450 content of brain is increased during pregnancy and after administration of ethanol, we examined whether 2D4 mRNA levels were affected. To study the effect of ethanol on 2D4 mRNA, male Wistar rats were injected with a single dose of ethanol (200 μl) intraperitoneally and sacrificed 3, 6, or 16 hr after injection. Total RNA derived from HPOA, cerebellum, cortex, olfactory bulbs, thymus, muscle, lung, prostate, and liver was subjected to Northern blot analysis using a 2D4-specific, radioactively labeled riboprobe recognizing the region between nucleotide position 156 and position 346 (Fig. 2). In liver a major band of the correct size (about 1800 nucleotides) could be detected, whereas in the various brain regions and lung two bands, one corresponding to the correct size and one corresponding to about 3000 nucleotides, were detectable. Further PCR studies indicated that an additional polyadenylation signal at positions 5625–5630 of the 2D4 genomic sequence (30) is used. This results in polyadenylation at position 5653, about

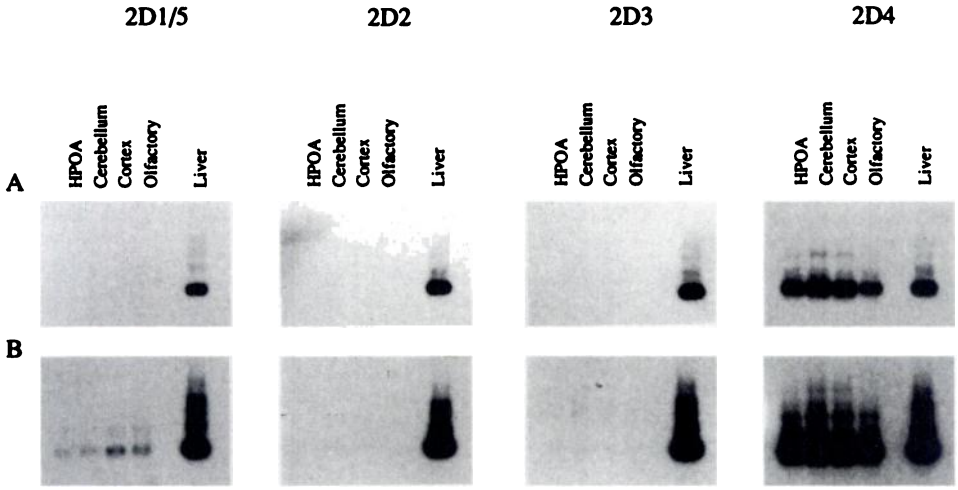

Fig. 1. RT-PCR amplification of 2D. Reverse-transcribed RNA from various brain regions (HPOA, cerebellum, cortex, and olfactory bulbs) and liver from Dark Agouti rats was used for PCR amplification. PCRs were performed with primers recognizing regions that are conserved within the rat 2D subfamily. Amplified products were separated on 2% agarose gels and transferred to nylon membranes. Membranes were probed with radiolabeled oligonucleotides specific for 2D1/5, 2D2, 2D3, or 2D4 and were exposed to Kodak X-Omat film for 2 hr (A) or 17 hr (B).

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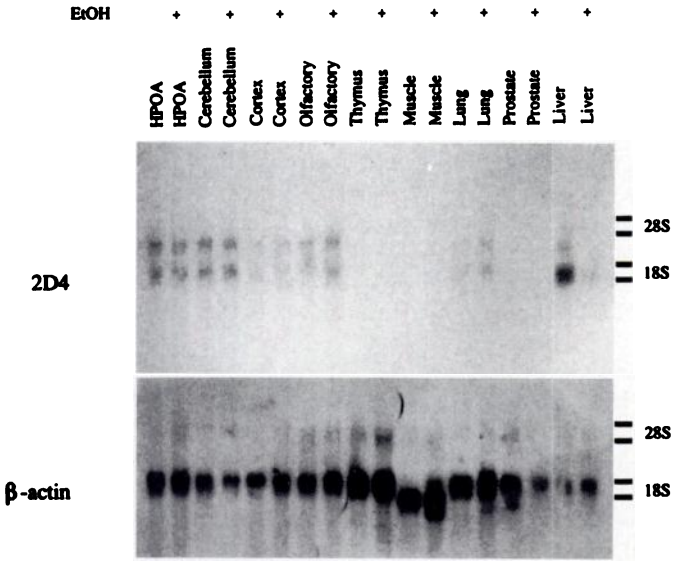

Fig. 2. Northern blot analysis of total RNA. Total RNA (20 μg) from various brain regions and organs from untreated and ethanol-treated (+*EtOH*) Wistar rats was subjected to Northern blot analysis and hybridized with 2D4-specific (A) and β-actin specific (B) probes. *Bars*, electrophoretic mobilities of 28 S and 18 S rRNA bands.

1000 nucleotides 3′ of the stop codon (data not shown). It is likely that the 3000-nucleotide band detected by Northern blot analysis is a differentially polyadenylated transcript, but to confirm this a 2D4-specific probe within the 3′ untranslated region will have to be used to probe the Northern blots. RNA derived from thymus, muscle, and prostate gave no detectable 2D4 signal. When duplicate blots were probed with a β-actin probe, no significant degradation of the RNA was evident.

Ethanol had no significant effect on brain or lung 2D4 mRNA levels (results shown in Fig. 2 for animals killed 16 hr after ethanol injection). In liver, however, there was a decrease in the 2D4 mRNA concentration after 16 hr. This was not evident 3 or 6 hr after ethanol treatment (data not shown).

Physiological regulation of 2D4 mRNA. The influence of age and hormonal status on brain 2D4 expression was also investigated. When brain regions and livers of pregnant and lactating animals were compared with controls, no significant difference in 2D4 mRNA signals on Northern blots was detected (data not shown). Because it has been shown that in rat liver the mRNA levels of all members of the subfamily except 2D4 are low at birth and increase with age (30, 31), we tested newborn rat brain for 2D4 mRNA expression. The expression level was similar to that in adult female rats, suggesting that there is no major postnatal developmental regulation at the 2D4 transcript level.

***In vitro* synthesis of 2D4 protein.** We synthesized 2D4 protein using a reticulocyte lysate transcription/translation system. *In vitro* synthesized 2D1v* served as a positive control. SDS-PAGE analysis of 2D1v* and 2D4 protein revealed that 2D4 protein had a greater electrophoretic mobility than did 2D1v* (Fig. 3). This difference in size could be due to a different conformation of 2D4, degradation of the protein, or use of an additional downstream, in-frame, translation initiation codon.

Antibody purification. To address the question of whether a stable protein is made from the 2D4 mRNA *in vivo*, specific antibodies were raised. Serum derived from a rabbit immunized with both a peptide corresponding to a 2D4-specific sequence (amino acids 108–119) and a peptide corresponding to a rat 2D conserved sequence (amino acids 284–295) was characterized by Western blot analysis of liver microsomes and immunoprecipitation of *in vitro* translated 2D1v* and 2D4 protein. The antiserum recognized one major band and a minor, faster migrating band of approximately 50 kDa on Western blots (Fig. 4A, *lane 1*). The major 50-kDa band signal could be almost completely eliminated by preadsorption of the antiserum with the peptide corresponding to the conserved region but not by the peptide corresponding to the 2D4-specific region (data not shown). On the other hand, *in vitro* translated 2D4 but not 2D1v* could be immunoprecipitated by the antiserum (data not shown). These results indicated that the antiserum contained two distinct populations of antibodies, one population against rat 2D P450s, which recognizes 2D proteins on Western blots but is not useful for immunoprecipitation, and one population against 2D4 protein, which can be used for immunoprecipitation. Both populations of antibodies were purified by affinity chromatography on the respective peptide-coupled Sepharose.

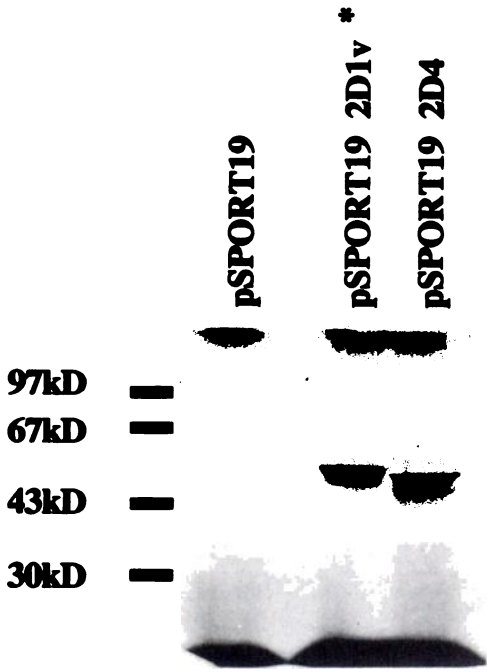

Fig. 3. *In vitro* transcription/translation of 2D1v* and 2D4. pSPORT19 vectors harboring no insert (pSPORT19), the 2D1v* cDNA insert (pSPORT19 2D1v*), or the 2D4 cDNA insert (pSPORT19 2D4) were used for *in vitro* transcription/translation. [^{35}S]Methionine-labeled proteins were separated on 7.5% SDS-polyacrylamide gels. Gels were exposed to Kodak X-Omat film for 12 hr. *Bars*, molecular weight standards.

Flow-through fractions and acid eluates were tested by Western blot analysis of liver microsomes and by immunoprecipitation of *in vitro* translated 2D4 protein. As shown in Fig. 4A, *lanes 2* and *4*, the 2D peptide column retained antibodies that recognized the major 50-kDa band. Antibodies in the flow-through fraction of the 2D4-specific peptide column recognized the major band (Fig. 4A, *lane 3*), whereas the eluate contained antibodies that recognized the faint lower band (Fig. 4A, *lane 5*). As a result of the purification, detection of the faint band was improved. Immunoprecipita-

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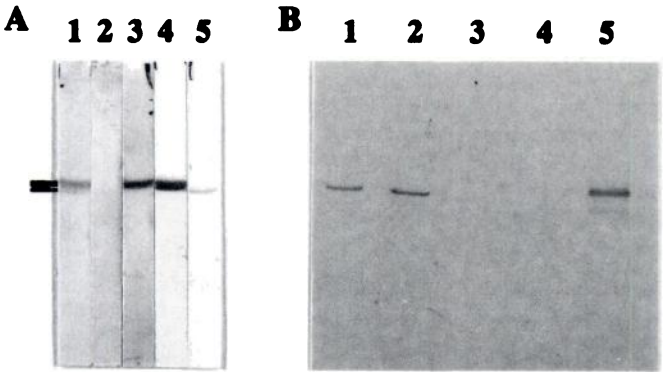

Fig. 4. Affinity purification of rat 2D- and 2D4-specific antibodies. A, Liver microsomes (40 pmol of P450/lane) were subjected to immunoblot analysis. Polyvinylidene difluoride strips were probed with unpurified serum (*lane 1*), the flow-through fraction of the conserved 2D peptide column (*lane 2*), the flow-through fraction of the 2D4-specific peptide column (*lane 3*), the acid eluate of the conserved 2D peptide column (*lane 4*), or the acid eluate of the 2D4-specific peptide column (*lane 5*). *Bars*, major and minor 50-kDa bands recognized by the antibodies. B, *In vitro* translated 2D4 protein was used for immunoprecipitation with unpurified serum (*lane 1*), the flow-through fraction of the conserved 2D peptide column (*lane 2*), the flow-through fraction of the 2D4-specific peptide column (*lane 3*), the acid eluate of the conserved 2D peptide column (*lane 4*), or the acid eluate of the 2D4-specific peptide column (*lane 5*).

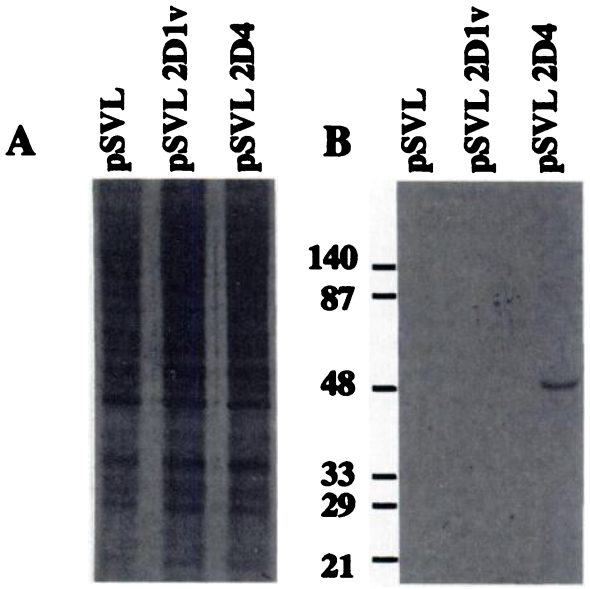

Fig. 5. COS-7 cell expression of 2D4 protein. COS-7 cells transfected with pSVL, pSVL 2D1, or pSVL 2D4 constructs were metabolically labeled with L-[^{35}S]methionine. A fraction of the cells were boiled in SDS sample buffer and analyzed by SDS-PAGE (A). The rest were used for immunoprecipitation with anti-2D antiserum. Immunoprecipitated material was analyzed by SDS-PAGE (B). *Bars*, molecular weight standards.

tion experiments with *in vitro* translated 2D4 protein showed that the 2D4-specific antibodies were useful for immunoprecipitation (Fig. 4B, *lane 5*) but those raised against the conserved 2D peptide were not (Fig. 4B, *lane 4*). These data suggest that the lower, faint, 50-kDa band recognized by the antiserum and the purified anti-2D4-specific peptide antibodies is 2D4 protein.

COS cell expression of 2D4 protein. We used COS-7 cell expression of 2D4 and 2D1v for the characterization of the purified anti-peptide antibodies. For this purpose we subcloned 2D4 and 2D1v (kindly provided by U. M. Zanger, Biocenter of the University of Basel, Basel, Switzerland) into the COS cell expression vector pSVL. We used the antiserum raised against the conserved 2D peptide and 2D4-specific peptide to test expression of 2D1v and 2D4 protein in COS-7 cells transfected with the respective pSVL constructs.

We were able to immunoprecipitate 2D4 but not 2D1v protein from transfected cells metabolically labeled with L-[^{35}S]methionine (Fig. 5). The 2D4 expressed in COS-7 cells showed the same electrophoretic mobility as the *in vitro* translated protein (data not shown). Although 2D mRNA was detectable in transfected COS-7 cells by Northern blot analysis (data not shown), we could not detect expression of 2D1v or 2D4 P450 in whole-cell lysates of these cells by Western blot analysis.

Western blot analysis of brain P450. Although the 2D mRNA was detectable in all brain regions tested, 2D4 protein was not detectable in brain microsomal fractions on Western blots, even when 100 μg of protein were loaded onto the gels. This indicated that the concentration of 2D4 was <10 pmol/mg of microsomal protein and that 2D4 was not a major form of P450 in brain. To quantitate the contribution of 2D4 to the overall content of P450 in brain, P450 was extracted from brain membranes, quantitated by its CO difference spectrum, and used in Western blots. A reproducible signal with the anti-2D4 antibody was obtained when 80 pmol of brain P450 were loaded in each lane (Fig. 6). When equal amounts of brain P450 from control and ethanol-treated rats were analyzed by Western blot analysis, similar signals were detected (data not shown). The faster migration, in the brain P450 fraction, of the protein recognized by the anti-2D4 antibody may reflect different migration in the SDS gels because of the presence of detergents used for solubilization of the P450. Alternatively, it could indicate that there is some degradation of the protein during the extraction from brain membranes. Degradation at the amino termini of several

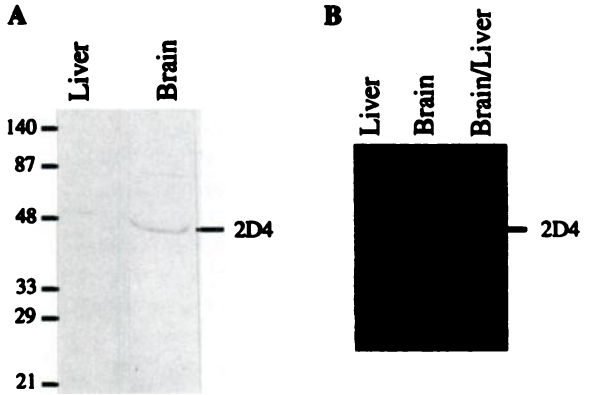

Fig. 6. Western blot analysis of brain P450 2D4. A, Liver microsomes (containing 40 pmol of P450) (*Liver*) and 80 pmol of brain P450 (*Brain*) derived from control rats were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes with liver and brain P450 were probed separately, under identical conditions, with purified anti-2D4 antibody and alkaline phosphatase-conjugated secondary antibody. B, Liver microsomes (containing 40 pmol of P450) (*Liver*), 60 pmol of brain P450 (*Brain*), and 60 pmol of brain P450 mixed with liver microsomes containing 20 pmol of P450 (*Brain/Liver*) were separated by SDS-PAGE. Western blot analysis was performed with goat anti-rabbit IgG-peroxidase conjugate as the secondary antibody. *Bars*, 2D4 bands.

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brain P450s has been observed previously (32). The amino terminus of 2D4 could not be identified in the brain P450 fraction because 2D4 represented a small fraction of the P450 loaded onto the gel. To determine whether the presence of the detergents in the brain P450 fraction could affect the mobility on SDS-polyacrylamide gels, the migration of 2D4 in liver microsomes was compared with that of liver microsomes mixed with brain P450. As shown in Fig. 6B, the migration of the band recognized by the anti-2D4 antibody was slower in the lane with liver microsomes than in the lane containing a mixture of brain P450 and liver microsomes. The mixture of liver and brain showed a single band, with electrophoretic mobility similar to that in the lane containing brain P450 only.

Discussion

Upon RT-PCR amplification, the most abundant 2D P450 mRNA in brain is that of 2D4, which is a minor form in liver. The detection of this mRNA by Northern blot analysis suggested that it might be an abundant P450 in brain. To determine what fraction of brain P450 could be accounted for by 2D4, we raised a specific peptide antibody to 2D4. With this antibody, P450 2D4 could not be detected by Western blot analysis of brain microsomes, even when 100 μg of microsomal protein were loaded in each lane. This result could mean either that no stable protein is expressed from the 2D4 mRNA in brain or that the concentration of 2D4 in brain is low. High levels of expression of a P450 mRNA with no accumulation of the corresponding protein have been convincingly demonstrated in the case of P450 2C13 in the livers of rats deficient in P450 2C13 (33). Because 2D4 has not been purified from the liver, one method for determining whether a stable protein could be produced from the mRNA was by cloning and *in vitro* translation of the cDNA. We cloned the cDNA from brain by PCR amplification and demonstrated the synthesis of a protein of 50 kDa when the 2D4 cDNA was translated in a reticulocyte lysate system. Furthermore, the anti-2D4-specific peptide antibody could immunoprecipitate the labeled protein. Transfection of COS cells with the 2D4 cDNA also resulted in the synthesis of an immunoprecipitable polypeptide of the correct size. Because a stable protein could be produced from the cDNA, we concluded that the lack of detection of the protein in brain microsomes was due to a low level of expression in the brain. To test the validity of this conclusion, P450 was extracted from brain and used in Western blots. The P450 content of the brains of untreated rats reported by most laboratories ranges between 2 and 10 pmol/mg of microsomal protein (20). Because the recovery of microsomes from brain homogenates by differential centrifugation is low, we routinely prepared brain P450 from a total membrane fraction. The yield of P450 was 30–50 pmol/g of wet weight.

When 80 pmol of P450 extracted from brain were used for Western blotting, a protein band of approximately 50 kDa was recognized by the antibody. With liver microsomes, a strong signal was obtained when 30 pmol of P450 were loaded in each lane. Because 2D4 represents <10% of the P450 in liver, we can estimate that it is possible to detect 3 pmol of 2D4. Hence, 2D represents <5% of brain P450. This explains why it is not possible to detect 2D4 in brain microsomes. It would be necessary to load >1 mg of brain microsomal protein to detect this protein.

The very low level of 2D4 protein in brain was confirmed with immunohistochemical studies (data not shown). There was an overall, very low level of expression in oligodendrocytes. This signal could be adsorbed out with the peptide that was used as the antigen but was not of high enough intensity to be photographed.

The substrate specificity of 2D4 remains to be determined. There are two steroid hydroxylases known to be distributed throughout the brain, i.e., 5α-androstane-3β,17β-diol hydroxylase (34) and estrogen 2- and 4-hydroxylase (35, 36). It is unlikely that 2D4 is identical to 5α-androstane-3β,17β-diol hydroxylase, because there was no detectable 2D4 mRNA in the prostate, a tissue in which 5α-androstane-3β,17β-diol hydroxylase is very abundant (34, 37).

No hydroxylation of estrogen could be demonstrated in COS cells after transfection of 2D4 cDNA. However, because the level of 2D4 expression was extremely low, no definitive statement can be made about catechol estrogen synthesis by 2D4. Attempts to overexpress this protein in *Escherichia coli* were unsuccessful.

The content of P450 in brain increases during pregnancy and lactation and upon ethanol treatment of rats. There was no detectable change in the 2D4 mRNA in rat brain under these conditions. However, when similar amounts of brain P450s from control and ethanol-treated rats were subjected to Western blot analysis, the 2D4 signal was approximately the same. This is different from the case of P450 2C and 2E1, where a clear induction of the protein could be seen after ethanol administration (32). Because the P450 content increased 2–5-fold, the amount of 2D P450 in brain must have increased, despite the lack of change in the mRNA level. A similar discrepancy between changes in the protein and mRNA levels was reported for P450 2E1 and 2C after ethanol treatment of rats (19). We conclude that under these conditions the P450 expression in brain is regulated at a post-transcriptional level. Whether regulation is at the level of the translation or the stability of the proteins remains to be determined. The anti-2D4 antibodies and cDNA probes will make it possible to resolve this issue.

A role for 2D P450s in the elimination of some endogenous or environmental neurotoxins involved in Parkinson's disease has been suggested. It is not known whether 2D P450 in the brain is important in this regard or whether the liver enzyme is sufficient for the elimination of toxins acting in the central nervous system. In an attempt to clarify this issue, the 2D P450s in brain have been examined. Of the five isozymes in this subfamily expressed in rats, the mRNA of 2D4, which is a minor form in liver, is the most abundant form in brain. The mRNAs of 2D2 and 2D3 were not detectable in brain. Due to the high degree of homology between 2D1 and 2D5, it is not possible to conclude from our study whether 2D1 or 2D5 mRNA, or both, is expressed in brain. However, because we were able to detect a signal in the brains of Dark Agouti rats, which do not express 2D1 mRNA in their livers, it seems likely that 2D5 mRNA is expressed in the brains of these rats. The importance of the different members of the 2D family in brain and their roles in neurodegenerative diseases will become clear only when their substrate specificities are established.

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