0026-895X/96/020342-09$3.00/0

MOLECULAR PHARMACOLOGY, 50:342–350 (1996).

Cytochrome P4502D4 in the Brain: Specific Neuronal Regulation by Clozapine and Toluene

EVA HEDLUND, ADRIAN WYSS, TOMMI KAINU, MARIA BACKLUND, CHRISTER KÖHLER, MARKKU PELTO-HUIKKO, JAN-ÅKE GUSTAFSSON, and MARGARET WARNER

Department of Medical Nutrition (A.W., M.B., J.-Å.G.) and Centre for Nutrition and Toxicology (E.H., M.W.), Karolinska Institute, S-141 86 Huddinge, Sweden, Hoffman-La Roche Ltd., Pharmacology Division, Preclinical Research, Basel, Switzerland (C.K.), Department of Neurology, University of Kuopio, Kuopio, Finland (M.P.-H.), and Department of Biomedical Sciences, University of Tampere, Fin-33101, Finland (T.K.)

Received January 17, 1996; Accepted April 26, 1996

SUMMARY

Twenty-four hr after a single dose of the neuroleptic drug clozapine, cytochrome P4502D4 (P4502D4) immunoreactivity, which was barely detectable in the brains of untreated rats, was clearly evident in neurons of the substantia nigra pars compacta, ventral tegmental area, granular neurons of the olfactory bulb, and Purkinje and granular neurons of the cerebellum. Induction was maintained with daily administration for 3 weeks. The mRNA for P4502D4 was detected by Northern blotting and localized by *in situ* hybridization in neurons throughout the brain and in the Bergman glia in the cerebellum. There were no detectable changes in the distribution or quantity of P4502D4 mRNA after treatment with clozapine. The overall P450 content of the brain increased with daily administration to a ~7-fold induction by 3 weeks of clozapine treatment. No induction of 2D4 was observed with the dopamine D_2 receptor blockers haloperidol, chlorpromazine, and sulpiride or with the serotonin receptor blocker mianserin. A clozapine-like induction of P4502D4 was obtained on administration of toluene to rats. The specificity of the induction of P4502D4 in the brain with respect to both the drugs that induce it and the cells in which it is induced suggests that induction of this enzyme could be involved in the therapeutic action of clozapine. The similarity of induction of P4502D4 elicited by clozapine and by the neurotoxin toluene suggests that more information is needed before a beneficial or toxicological role can be assigned to this isozyme.

The etiology of degenerative diseases of the central nervous system is unknown, but there is a growing body of evidence indicating the involvement of environmental factors. The agents most commonly mentioned in this regard are metals, organic solvents, reactive oxygen metabolites, and pesticides (1, 2). The discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine can induce parkinsonism (3) clearly shows that xenobiotics can be activated within the CNS to metabolites that cause neuronal degeneration.

Several members of the P450 superfamily of mixed-function oxidases (4) have been well characterized for their ability to metabolize pharmaceuticals and to cause cytotoxicity and mutagenesis through the generation of reactive metabolites of chemicals and oxygen (5, 6). The production of such cytotoxins within neurons, even at low levels, could lead to cumulative damage over time and result in a shortened life span of the neurons. Convincing evidence of a role of brain P450 in chemically induced neurotoxicity, age-related degeneration of the CNS, or pharmacological action of therapeutic agents has not yet been obtained because of the low level of the enzyme in the brain and the difficulty of identifying the cells that harbor the enzymes. The concentration of P450 in the brain of untreated rats is <1% of that in the liver. In most studies, hepatic forms account for a minor fraction of the P450 in the brain (for a review, see Ref. 7). Members of the 2D subfamily have been detected in the human, canine (8), and rat (9, 10) brain. This subfamily is of interest in brain pharmacology and toxicology because it is responsible for the metabolism of many CNS active drugs such as dopaminergic antagonists, adrenergic antagonists, antidepressants, and analgesics (11) and because in humans there seems to be a link between defects in the 2D gene and Parkinson's disease (12–14).

In rats, the P450 content and isozyme profile of the brain can be altered by drugs and environmental contaminants. Ethanol, for example, induces several hepatic forms of P450 in the brain (15), and such induction probably increases the burden of reactive metabolites formed from xenobiotics and oxygen within the brain. Toluene is another solvent that is

This work was supported by grants from the Swedish Medical Research Council (13X-06807 and B92-03P-08561-04A), Swedish Work Health and Environment Fund, and Hoffmann-La Roche Basel.

ABBREVIATIONS: CNS, central nervous system; P450, cytochrome P450; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; IR, immunoreactivity; BSA, bovine serum albumin; PCR, polymerase chain reaction.

associated with CNS toxicity. There are numerous reports of the acute and chronic toxic effects of toluene on the CNS as well as the combined toxicity of toluene with other solvents such as *n*-hexane (for reviews, see Refs. 16 and 17). Toluene has a high abuse potential, and its abuse is associated with severe cerebral cortex, cerebellar, and auditory toxicity (16). In the liver, toluene induces several forms of P450, including 2E1, 2B, and 3A (17).

Neuroleptic drugs represent one class of clinically useful drugs whose use is associated with the development of neurotoxicity. Extrapyramidal dysfunction is frequently observed with chlorpromazine and haloperidol, two neuroleptics whose therapeutic effect is correlated with their ability to block dopamine D_2 receptors. Clozapine, an atypical neuroleptic, has a very low affinity for the dopamine D_2 receptor but a high affinity for dopamine D_4 and serotonin receptors. Its use is not associated with extrapyramidal side effects (18–21).

We examined whether neuroleptic drugs and the solvent toluene influence P450 in the brain. These studies are a prelude to the study of whether the toxicological and/or pharmacological effects of these agents are related to the P450 content, distribution, or isozyme profile in the brain.

Materials and Methods

We obtained anti-1A and -2B from T. Haaparanta (Karolinska Institute, Huddinge, Sweden); anti-1A1 and -1A2 peptide antibodies, R. J. Edwards (Royal Postgraduate Medical School, London, UK); anti-2C, A. Mode (Karolinska Institute, Huddinge, Sweden): anti-2E1, Oxygene (Dallas, TX); anti-3A, A. Åstrom (Stockholm University, Stockholm, Sweden); and anti-4A, G. Gibson (University of Surrey, Surrey, UK). 2D4 antibodies were raised in the laboratory (Karolinska Institute, Huddinge, Sweden) as described previously (9).

Animals. Male Sprague-Dawley rats (180–200 g) were obtained from Møllegaard (Ejby, Denmark). They were kept under controlled conditions of temperature and humidity and had free access to food and water. All drugs were administered daily by gavage for 1–3 weeks at doses of 1 mg/day/rat for clozapine, mianserin, and sulpuride and 0.2 mg/day/rat for haloperidol. Toluene was administered as a single intraperitoneal dose of 100 μl. In addition, rats were exposed to toluene by inhalation at 140 ppm for 3 hr on three consecutive days. Animals were killed 24 hr after the last dose or exposure. Sixteen rats were used for the immunocytochemical and *in situ* hybridization studies with toluene. Eight of the animals were injected with toluene (250 mg/kg body weight) 24 hr before death, and the other eight were used as controls. For studies with clozapine, three rats were given a single oral dose of clozapine 24 hr before death.

Toluene inhalation. For inhalation experiments, rats in their cages were housed in solvent chambers for 3 days before solvent exposure. Controls were treated exactly as were exposed rats except that no solvent was passed into their chambers. Toluene, contained in a flask outside of the chambers, was blown into the sealed chambers with a stream of air. The concentration of toluene in the chamber was monitored with an on-line IR spectrometer, and the toluene concentration was adjusted by regulating the flow of air through the toluene flask. In these experiments, the toluene concentration was kept at 140 ppm, and the exposure time was 3 hr. Rats were exposed once a day on three consecutive days.

For immunocytochemical studies, the animals were perfused through the ascending aorta while under chloral hydrate anesthesia with 100 ml of saline. This was followed by a 3-min perfusion with a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M PBS. Brains and livers were excised and further fixed with immersion for 60 min. After cryoprotection with 10% sucrose, the tissues were frozen on a block of dry ice. For *in situ* hybridization studies, the animals were decapitated, and the tissues were extracted and frozen. Subsequently, 14-mm-thick coronal and sagittal sections of the brains and sections of the livers were cut with a Microm HM500 cryostat. Sections were thawed onto chromogelatin-subbed glass slides for immunohistochemical studies and onto Prob-on glasses (Fisher Scientific, Pittsburgh, PA) for *in situ* hybridization studies.

For P450 isolation, rats were anesthetized with carbon dioxide and decapitated. Brains and livers were removed immediately and placed in ice-cold PBS containing 20% glycerol and 1 mM EDTA. For RNA isolation, tissue was homogenized in guanidinium thiocyanate buffer (22) frozen in liquid nitrogen and stored at −70° until use.

Immunocytochemical studies. Several sections representing different brain areas and sections of liver were processed to demonstrate P4502D4 IR. The sections were incubated for 48 hr at 4° with purified 2D4 antibodies (1:5 dilution). This was followed by biotinylated goat anti-rabbit IgG and the ABC complex (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen to visualize the P4502D4 IR. All of the antibodies were diluted in PBS containing 1% BSA and 0.3% Triton-X 100. After staining, the sections were dehydrated and embedded in Entellan. The sections were photographed with a Nikon Microphot FXA microscope. Controls included omission of the primary and secondary antibodies, staining with preimmune rabbit serum (1:5 dilution) and antiserum preabsorbed with the peptide used for preparation of the antibody. The antibody was preabsorbed for 24 hr with the peptide at a concentration of 10^{-5} M. None of the IRs described below were observed in the controls.

***In situ* hybridization studies.** Four 2D4-specific antisense oligonucleotide probes were used for *in situ* histochemical studies: (1) complementary to nucleotides 226–265, 5′ CTGGCAGCCCATTCAGTACAACCACCGACTCAAAGGCCAG 3′; (2) complementary to nucleotides 501–540, 5′TAGGAGTGTTAGGGCTGAAAGGGAATCCACTATGGTCAGCG 3′; (3) complementary to nucleotides 620–659, 5′ GATTCCTCCTCAAGAGTGTCCTTCAGCAAGTCCAGGAGCC 3′; and (4) complementary to nucleotides 660–699, 5′ GAGCATCGGGAACACATTCAGGAGCATGGGCAGGAATCCA 3′.

The sequences exhibited <60% homology with any other gene in the GenBank database. Also, several control probes of the same length, similar in GC content and specific activity, were used to determine the specificity of the hybridizations. The probes were labeled with [α-^{33}P]ATP (New England Nuclear, Boston, MA) using terminal deoxynucleotidyltransferase (Amersham International, Buckinghamshire, UK) to a specific activity of 6×10^8 cpm/mg. The sections were briefly air dried and hybridized at 42° for 18 hr with 10×10^6 cpm/ml of the probe in a mixture containing 4× standard sodium citrate, 50% formamide, 1× Denhardt's solution, 1% sarkosyl, 0.02 M phosphate buffer, pH 7.0, 10% dextran sulfate, 500 mg/ml heat denatured salmon sperm DNA, and 200 mM dithiothreitol. After hybridization, the sections were rinsed four times at 55° in 1× standard sodium citrate (1 × = 8.75 g/liter NaCl and 4.41 g/liter sodium citrate, pH 7.0) for 15 min each and subsequently left to cool for 1 hr at room temperature. The sections were dipped in distilled water, dehydrated with 60% and 90% ethanol, and air dried. Then, the sections were covered with Amersham β-max autoradiography film. After 30 days' exposure, the films were developed using LX24 developer and AL4 fixative (Eastman Kodak, Rochester, NY). The films were then analyzed with a Dage-MTI CCD-72 camera (Dage-MTI, Michigan City, IN) connected to a Quick Capture frame grabber board (Data Transmission, Marlboro, MA). The mean densities of the different brain areas and sections of the livers were analyzed with Image 1.16 software (National Institutes of Health, Rockville, MD) calibrated with Amersham ^{14}C standards. Alternatively, the sections were dipped in NTB2 emulsion (Eastman Kodak) diluted 1:1 with distilled water and exposed at −20° for 50 days. The sections were

dipped with D19 developer (Kodak), fixed with G333 fixative (Alga Gevaert, Leverkusen, Germany), and coverslipped with a mixture of glycerol and PBS (3:1).

Isolation of total RNA. Total RNA was isolated according to the guanidinium thiocyanate single-step method described by Chromczynski and Sacchi (22).

Northern blot analysis. RNA 20-μg aliquots 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′ ATCATGAAGCTT AGACTTCCAGAATATGCCAGCGG 3′) and antisense (5′ TCTCACGGATCC CAAAGCCCGACTGGTCATTGAAA 3′) primers designed with a *Hind*III (5′ ATCATGAAGCTT 3′) and a *Bam*HI (5′ TCTCACGGATCC 3′) restriction site, respectively, were used for reverse transcription-PCR of brain total 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; 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 0.5 mM concentration of ATP, GTP, and CTP; 100 μCi of [α-^{32}P]UTP (800 Ci/mmol); 40 units of RNasin RNase inhibitor; 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.

Hybridization with 2D4 riboprobe. Membranes were prehybridized in 6× standard sodium citrate (0.9 M NaCl, 0.09 M sodium citrate), 1% BSA, 1% SDS, 50% formamide, and 200 μg of salmon sperm DNA/ml 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 [α-^{32}P]UTP-labeled 2D4 riboprobe. Filters were washed three times for 5 min with 2× standard sodium citrate/1% SDS, twice for 5 min and once for 90 min with 0.1× standard sodium citrate/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 (Amersham).

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

Preparation of brain P450. Brain P450 was prepared from solubilized brain total membrane preparation as described previously (15). A 20% (w/v) homogenate in PBS containing 1 mM EDTA and 20% glycerol was prepared with a polytron. Phenylmethylsulfonyl fluoride (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. Phenylmethylsulfonyl fluoride 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 chromatographed on a column (5 × 2.5 cm) of *p*-chloroamphetamine-coupled Sepharose as described previously (15). The column was washed with 100 ml of 1/4 diluted solubilization buffer, and P450 was eluted with solubilization buffer. P450 was quantified spectrally (25), and aliquots were taken for Western blots. Proteins were precipitated with chloroform/methanol and resolved on 10% SDS-polyacrylamide gels according to the method of Laemmli (26). Then, 40 pmol of P450, equivalent to 2 μg of P450 protein, was applied to each lane.

Western blot analysis. After SDS-polyacrylamide gel electrophoresis, proteins were transferred onto Pro Blot membranes on a semidry blotter (1.2 mA/cm^2 membrane for 2 hr) or in buffer in 125 mM Tris/960 mM glycine 250 mA for 1 hr. Horseradish peroxidase-conjugated goat anti-rabbit IgG at a dilution of 1:3000 was used as the secondary antibody. Blots were blocked by an overnight incubation at 5° in 10% fat-free milk in PBS containing 0.1% Nonidet P-40, and incubations with primary and secondary antibodies were done in 10% fat-free milk in PBS containing 0.1% Nonidet P-40. Bands were visualized with enhanced chemiluminescence (ECL, Amersham)

Results

Induction of brain P450 by neuroleptics and toluene. Sulpuride, mianserin, and clozapine were good inducers of brain P450, causing a 3–6-fold increase, but haloperidol had no effect on the level of P450 in the brain. The effect of clozapine was evident after 1 week of treatment, and the brain P450 content increased further with treatment for 2 and 3 weeks (Table 1). Toluene, when given intraperitoneally, increased the level of P450 in the brain 3–4-fold within 24 hr. Inhalation of toluene also resulted in a 4-fold induction of brain P450.

Identification of induced P450s by Western blotting. To identify the P450s induced, Western blots were done with the following panel of antibodies: two anti-peptide antibodies against P450s 1A1 and 1A2; an antiserum raised against purified P4501A1 that recognizes both 1A1 and 1A2; an antiserum raised against purified 2B1; an antiserum raised against 2C11 that recognizes 2C11, 2C12, 2C13, and 2C7; a peptide antibody that recognizes all members of the rat 2D subfamily, 2D1, 2D2, 2D3, 2D4, and 2D5; a peptide antibody specific for 2D4; an antiserum raised against purified 2E1; an antiserum raised against purified 3A1 that recognizes 3A1 and 3A2; and a sheep antiserum that recognizes 4A1, 4A2, and 4A3.

As reported previously (27), P4504A was present in the brains of all rats, and the level of this enzyme remained constant in both treated and control rats. The only other form of P450 that was identified was 2D4. The level of P4502D4 was increased in the brain after clozapine but not after haloperidol treatment (Fig. 1, *left and middle*). Because the D2 receptor blocker haloperidol did not induce 2D4 in the brain, it seemed unlikely that the induction of 2D4 could be related to D_2 receptor blockade. We therefore compared the effect of clozapine with that of mianserin, a serotonin receptor blocker, and sulpuride, a blocker of both dopamine D_2 and serotonin receptors. Of these drugs, only clozapine induced P4502D4 in the brain (Fig. 1, *right*).

All of the control brain P450 in Fig. 1 comes from the same pool of brain P450; this is because there is some variability in the amount of P4502D4 in the brains of untreated rats. We examined three separate pools of brain P450 prepared from the brains of control rats. There were 10 rats in each pool. Forty picomoles of P450 from each pool was loaded onto gels, and blots were probed for P4502D4. There was variability in the P4502D4 content even though the yield of P450 from each

TABLE 1

Changes in P450 in the brain and liver after administration of neuroleptics and toluene

Treatment	P450 content of the brain: wet weight	P450s identified in brain	P450s induced in liver
	pmol/g		
Control (mean ± SD, n = 14)	36 ± 13	4A, 2D4	
Control olfactory lobes	30	4A, 2D4	
Toluene (intraperitoneal)	150	4A, 2D4	2E1, 2D4, 2B, 3A
olfactory lobes	350	4A, 2D4	
Toluene exposure by inhalation	98, 82, 120	4A, 2D4	nd[a]
Clozapine			
24 hr	30	4A, 2D4	
1 week	84, 90	4A, 2D4	2B
2 weeks	130	4A, 2D4	1A, 3A, 2B
3 weeks	173, 161, 212	4A, 2D4	1A, 3A, 2B
Haloperidol			
1 week	>20, 31	4A	2B
3 weeks	35, 34	4A	2B
Sulpiride 1 week	99	4A, 2D4	nd[a]
Mianserin 1 week	101	4A, 2D4	nd[a]
Control for handling of rats			
Daily gavage with sucrose solution 2 weeks	17	4A, 2D4	
Daily gavage with sucrose solution 3 weeks	48	4A, 2D4	

[a] nd, not done.

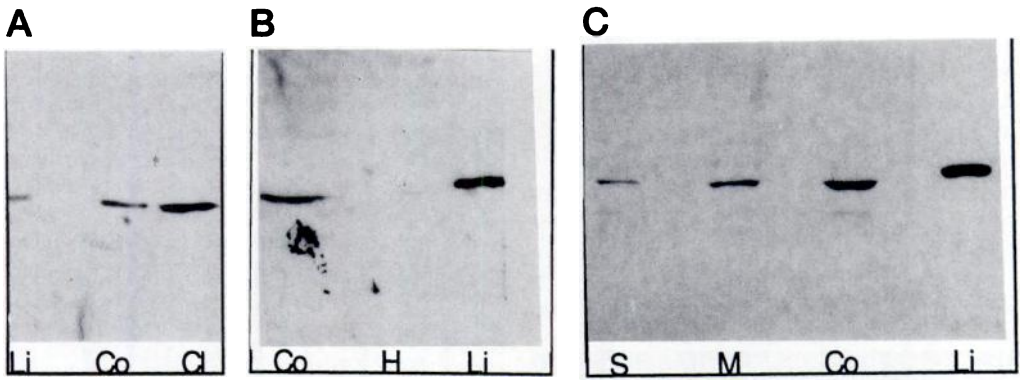


Fig. 1. Detection of P4502D4 in the brain by Western blot analysis of brain P450 after neuroleptic drug treatment. *Left*, comparison of the P4502D4 signal in the P450 fraction from the brains of control (*Co*) and clozapine-treated (*Cl*) rats. *Middle*, P4502D4 in the brain P450 fraction from control (*Co*) and haloperidol-treated (*H*) rats. *Right*, P450 fraction from sulpuride-treated (*S*), mianserin-treated (*M*), and control (*Co*) rats. Forty picomoles of brain P450 and 40 pmol liver P450 (*Li*) were loaded in the respective lanes. For analysis shown on *left*, exposure time of the film was 1 min. For other analyses, exposure time was 5 min.

pool was not different. We chose the pool with the highest 2D4 signal as our standard of brain P450; this is the pool that is used in all comparisons. The apparent decrease in P4502D4 in the brain after treatment of rats with the dopamine D_2 receptor blockers haloperidol and sulpuride may simply be a result of our having used the highest control values as our standards.

After toluene treatment, the overall P450 content of the brain increased 3–4-fold, and in the olfactory lobes, the increase was ~10-fold (Table 1). Except in the olfactory lobes, where there was a large induction of P4502D4 (Fig. 2), most of the P450 in brain after toluene treatment was not P4502D4. This is clearly evident from the Western blots, which revealed that when similar amounts of P450 from the brains of control or toluene-treated rats were loaded onto the gels, the intensity of the signals was similar. Because there is 3–4-fold more P450 in the brain after toluene treatment, 2D4 must have increased, but it nevertheless remained at ~1% of the brain P450. Some other form or forms of P450 must have increased by toluene treatment.

Because of the induction of this enzyme by both clozapine and toluene, the mechanism of this induction and the cells in which the induction occurred were further investigated.

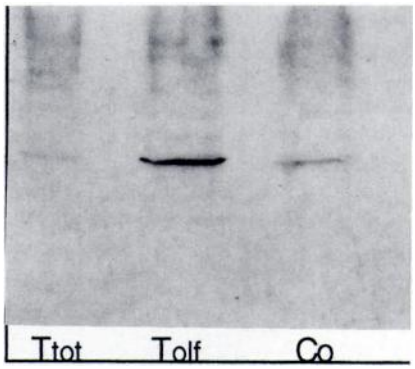


Fig. 2. Western blot of brain P450 after toluene treatment. Twenty-four hr after a single dose of toluene, P450 was prepared from the olfactory lobes (*Tolf*) and whole brain (*Ttot*); 40 pmol P450 was loaded onto the lanes and compared with 40 pmol from control brains (*Co*). The blot was probed with anti-2D4 antibodies. Exposure time of the film was 1 min.

Distribution of P4502D4 mRNA in the brain. *In situ* hybridization revealed that the mRNA for P4502D4 was dis-

tributed throughout the brain of control rats (Fig. 3a). There were no consistent and reproducible changes in the quantity or distribution of the mRNA after treatment with toluene (Fig. 3b) or clozapine (data not shown). The strongest expression of 2D4 mRNA was observed in the Bergman glia and granular layer of the cerebellum. There also was staining of the superior and inferior colliculi, inferior and superior olivae, facial nucleus, and pontine nucleus. Weaker expression was seen in the olfactory bulbs, ventral tegmental area, and substantia nigra. Glial cells that harbored the 2D4 mRNA were evident in the olfactory bulb, inferior colliculus, cerebellar penduncles, and pontine track. Signals were also present in the ependymal lining that covered the lateral ventricles and in the basal cell layer of the forebrain. The apparent differences between control and toluene-treated brain in Fig. 3 reflect differences in the sections and are not due to the treatment.

Northern blots confirmed the distribution observed with *in situ* hybridization; i.e., the level of 2D4 mRNA was higher in the cerebellum than in other areas of the brain and there were no significant changes in the level of 2D4 RNA in the brain after toluene or clozapine treatment (Fig. 4). As reported previously, there are two 2D4 transcripts due to the use of two different polyadenylation signals (9).

Immunohistochemical localization of P4502D4 in the rat brain. Immunohistochemical staining of the brains of untreated rats for P4502D4 revealed a strong signal only in the basal cell layer of the forebrain. Very weak staining was observed in neurons (granular cells of the internal granular layer) of the olfactory bulb, substantia nigra, ventral tegmental area, and Golgi neurons of the cerebellar cortex (Fig. 5, a, d, and g). After toluene treatment (Fig. 5, b, e, and h), there was a clear increase in the number of labeled neurons in the granular cells of the olfactory bulb, pars compacta of the substantia nigra, and ventral tegmental area. In the cerebellum, Purkinje neurons and cells in the granular layer were intensely stained.

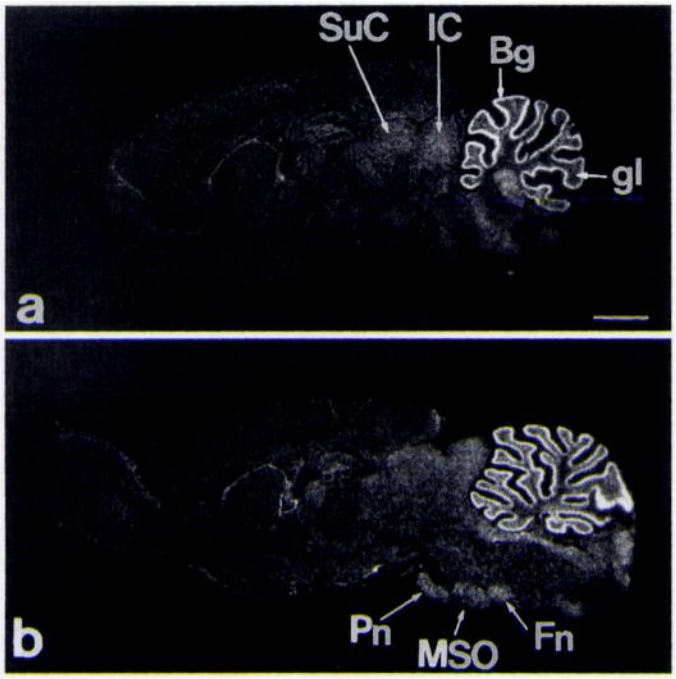


Fig. 3. *In situ* hybridization of 2D4 mRNA in the brains of control (a) and toluene-treated (b) rats. *Bg*, Bergman glia; *gl*, granular layer of the cerebellum; *IC*, inferior; *SuC*, superior colliculus; *Pn*, pontine nucleus; *MSO*, medial superior olive; *Fn*, facial nucleus. Staining of multiple sections revealed that there is no clear and consistent induction of 2D4 mRNA after toluene treatment in any brain region. *Bar*, 2.5 μm.

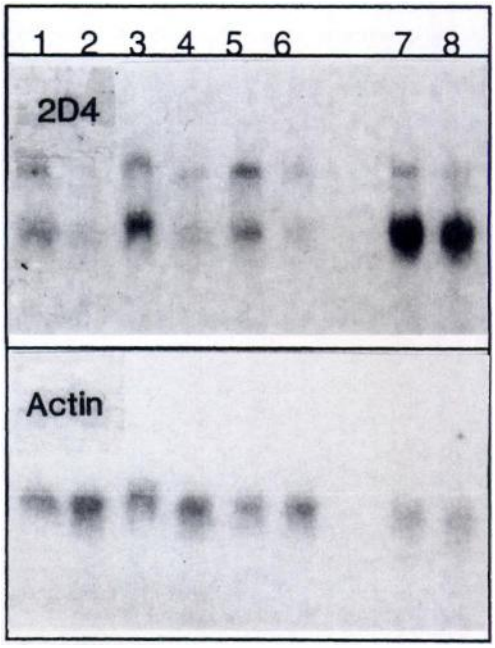


Fig. 4. Northern blot analysis of total RNA. Total RNA (20 μg) from cerebellum and olfactory lobes of control (*lanes 1 and 2*), toluene-treated (*lanes 3 and 4*), and clozapine-treated (*lanes 5 and 6*) rats were subjected to Northern blot analysis and hybridized with a 2D4- and a β-actin-specific probe. *Lanes 1, 3, and 5*, cerelellum; *lanes 2, 4, and 6*, olfactory lobes; *lanes 7 and 8*, results with RNA from livers of control and toluene-treated rats, respectively.

At 24 hr after a single dose of clozapine (Fig. 5, c, f, and j), the number of labeled neurons was greatly increased in granular cell layers of the olfactory bulbs and cerebellar cortex, Purkinje cells of the cerebellar cortex, ventral tegmental area, and pars compacta of the substantia nigra. The supraoptic nucleus also contained numerous moderately stained cells. All staining was completely absorbed by preincubation of the antibody with the peptide used for immunization.

Induction of P450 in the liver by clozapine and toluene. In the liver, both 2D4 and its mRNA were present in the control animal (Fig. 6). The mRNA showed a diffuse expression (Fig. 6a), whereas the immunohistochemical study revealed a few strongly stained hepatocytes that were scattered throughout the liver (Fig. 6b). There was a modest increase in the amount of 2D4 mRNA after toluene treatment (Fig. 6a) and a prominent increase in the number of 2D4 IR hepatocytes (Fig. 6c). The positively stained cells were clustered in distinct patches, and the staining was strong. The 2D4 level in the liver was not affected by clozapine (Fig. 6d). Staining of the toluene-treated liver was completely abolished by preabsorption of the antibody with the peptide against which it was raised (Fig. 6e).

The mechanisms of induction of P450 in the liver by clozapine and toluene seem to be different because toluene but not clozapine was a good inducer of 2D4 (Figs. 7 and 8) in the liver and because both clozapine and toluene induced forms of P450 other than 2D4 in the liver that were not induced in

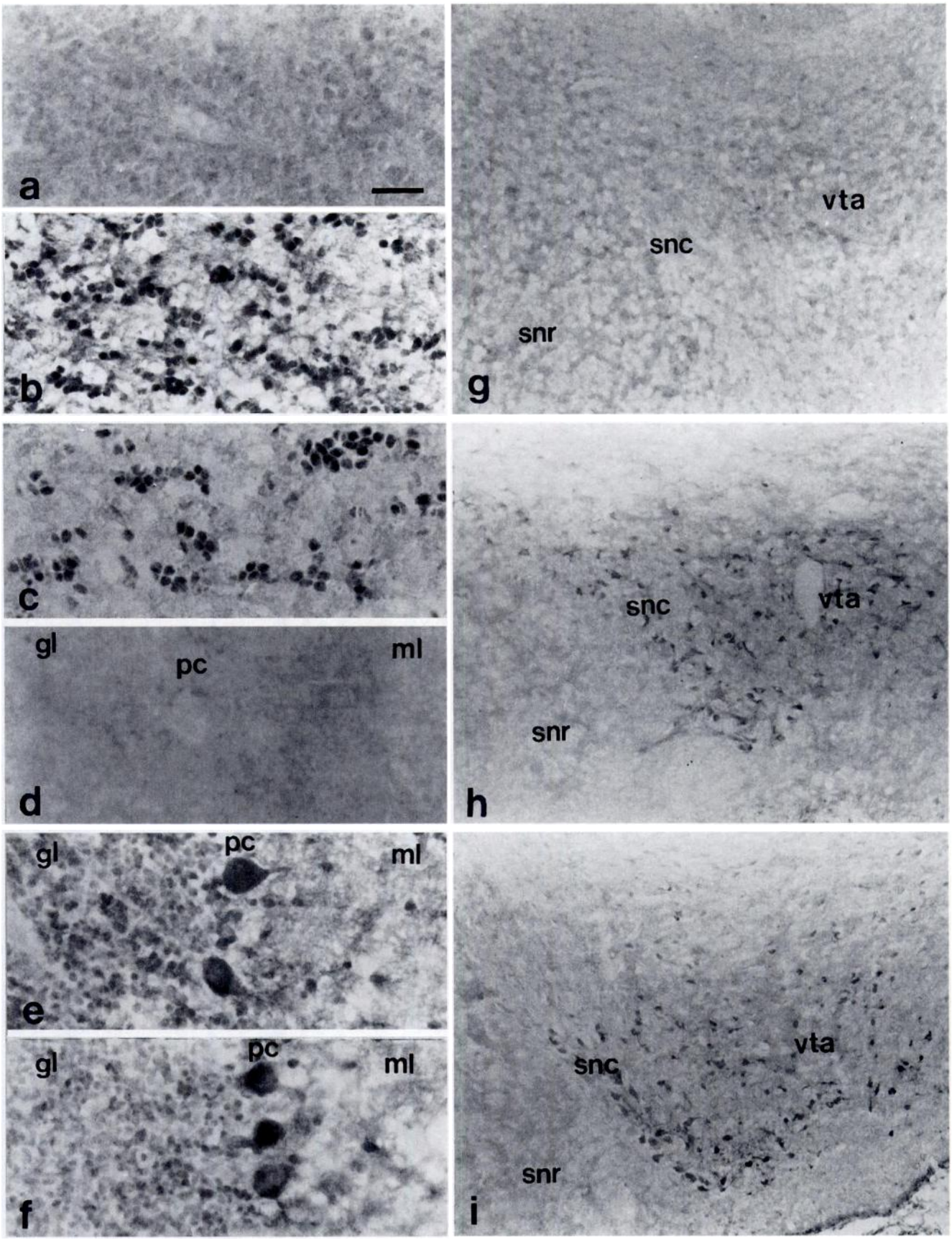
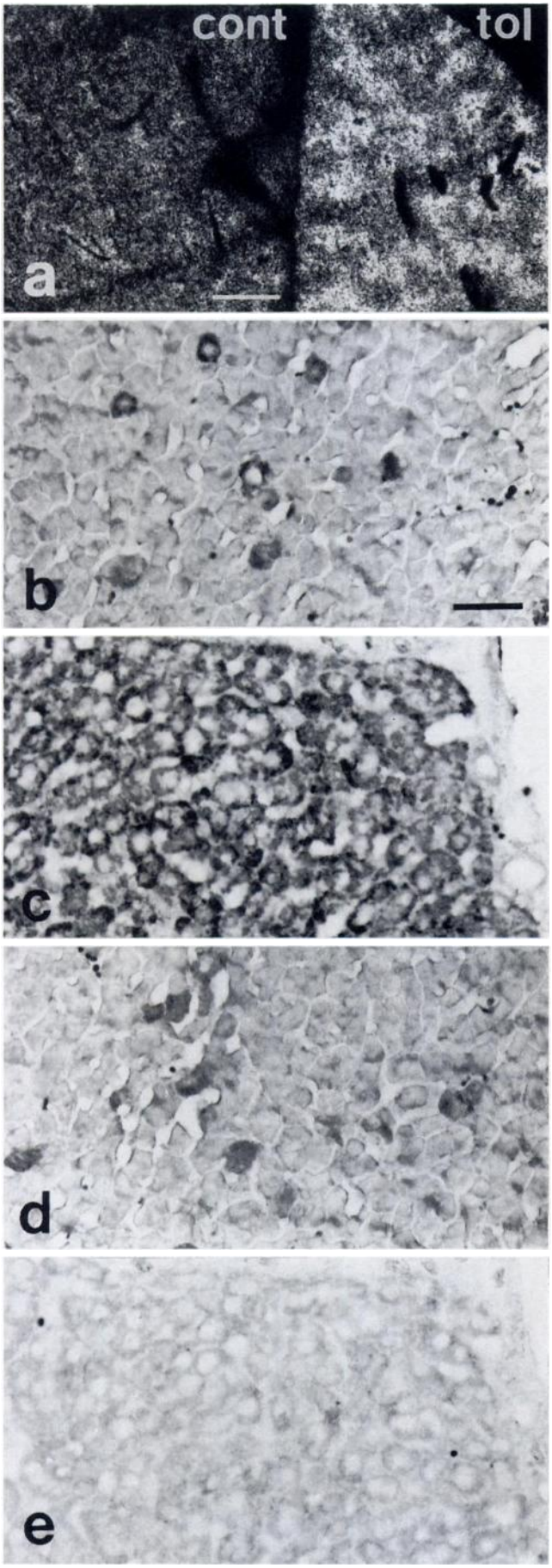


Fig. 5. Immunocytochemical demonstration of P4502D4 in the rat brain. a–c, Olfactory lobes of control (a), toluene-treated (b), and clozapine-treated (c) rats. d–f, Cerebella of control (d), toluene-treated (e), and clozapine-treated (f) rats. *gl*, granular layer; *pc*, Purkinje cell layer; *ml*, molecular cell layer. g–i, Ventral tegmental area (*vta*), substantia nigra compacta (*snc*), or substantia nigra reticularis (*snr*) of control (g), toluene-treated (h), and clozapine-treated (i) rats. *Bar*, 50 μm for a–c, 25 μm for d–f, and 100 μm for g–i.



the brain. For clozapine, these forms of P450 were 3A, 1A, and 2B (Fig. 7). For toluene, as previously shown (17), the forms were 2B, 2E1, and 3A.

Discussion

We have shown that the neuroleptic drug clozapine and the solvent toluene are good inducers of P450 in the brain, increasing the overall P450 content of the brain 3–6-fold over that in untreated animals. One of the forms of P450 induced is 2D4. Neither agent caused significant changes in the level or distribution of 2D4 mRNA in the brain, but there is specific and selective neuronal induction of the 2D4 protein by each agent. The mechanisms involved in the neuronal induction of 2D4 protein are not known. Post-transcriptional increase seems to be a common mechanism of increasing the P450 content of the brain. It was also observed with the induction of multiple forms of P450 in the brain after ethanol administration (15, 28) and in the regulation of the expression of P450 aromatase in the brain (29). This may be a mechanism through which rapid increases in the proteins can be achieved in the brain. If post-transcriptional regulation is a general mechanism for induction of brain P450s, elucidation of the role of brain P450 in the development of tolerance to drugs, in chemically induced neurotoxicity, and in the therapeutic and/or toxic effects of neuroactive drugs will be a much more difficult task because it will be not be sufficient to measure changes in mRNA levels.

Interestingly, in several brain areas in which the mRNA for 2D4 is expressed, neither clozapine nor toluene increased the level of the 2D4 protein. It can be anticipated that other agents will be found that affect the 2D4 level in the these regions in which the 2D4 mRNA level is high (i.e., neurons of the hippocampus, inferior colliculus, superior olive, pontine nucleus, and Bergman glia of the cerebellum).

Toluene is a neurotoxin that causes cerebellar and cerebral cortex atrophy and auditory impairment (16). The mechanisms involved in these toxicities are unknown. In the current study, we demonstrated that inhalation of toluene at a concentration of 140 ppm, a value set by the Swedish Occupational Health and Safety Administration as the threshold limit for exposure of humans, increases the level of P450 in the rat brain. This level of exposure to toluene has been shown to decrease the affinity of dopamine and serotonin for their respective receptors (32). This effect of toluene is thought to be due to changes in membrane fluidity because it can be blocked by the ganglioside GM1. In our study, toluene exposure resulted in a 4-fold induction of P450 in the brain. P4502D4 represents a small fraction of the induced P450, but its induction in specific neurons in the cerebellum and inferior olivae, areas associated with the toxicity of toluene, suggests that a careful examination of the induction of P450 in neurons is warranted. One possible explanation for the very rapid increase in P4502D4 after a single dose of toluene is that 2D4 is one of the P450s involved in protection of the

Fig. 6. a, *In situ* hybridization of P4502D4 mRNA from livers of control (*cont*) and toluene-treated (*tol*) rats. b–d, Immunochemical evidence of P4502D4 in liver of untreated rats (b), toluene-treated rats (c), and clozapine-treated rats (d). 2D4 immunostaining is completely abolished after preabsorption of the antibody with the peptide used for immunization. This is shown for the toluene-treated liver (e). *Bars*, 1 (a) and 200 μm (b–e).

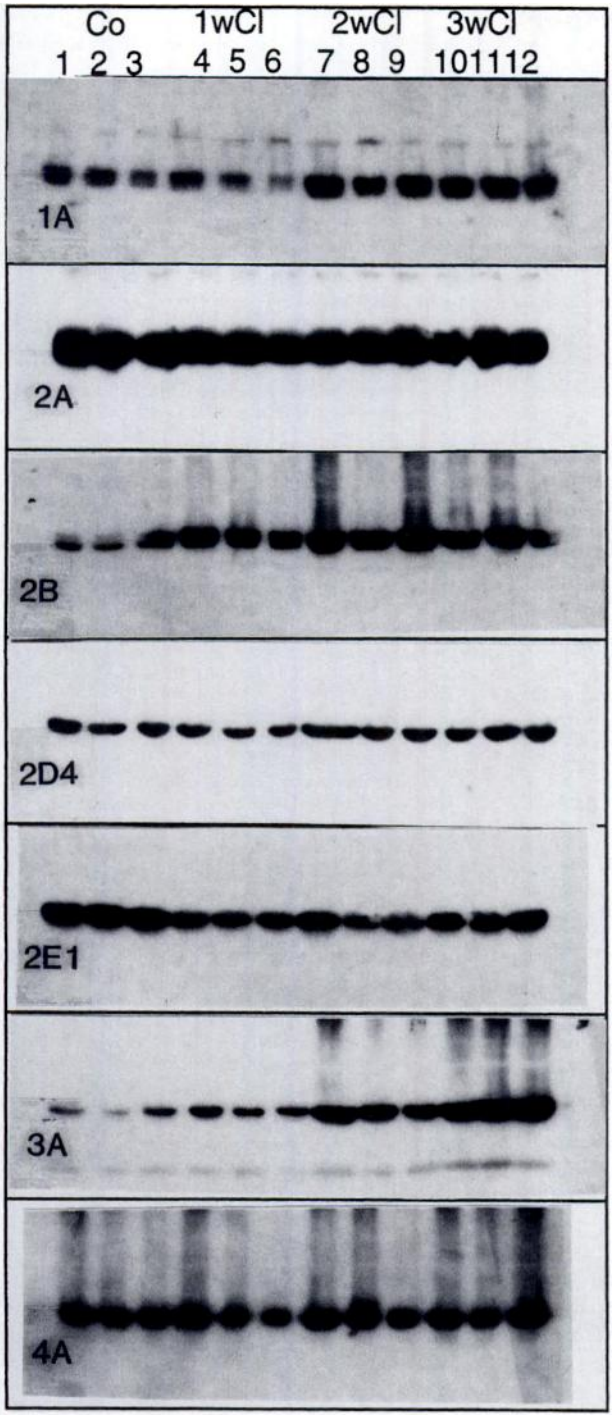


Fig. 7. Western blot analysis of liver cytochrome after clozapine treatment. Each lane contains liver microsomes (10 pmol of P450) from control rats (*Co*) or rats that were treated with clozapine for 1 (*1wCl*), 2 (*2wCl*), and 3 (*3wCl*) weeks. Blots were probed with antibodies against P450s 1A, 2A, 2B, 2D4, 2E1, 3A, and 4A. The experiment was done with three animals in each treatment group, and each lane represents microsomes from an individual rat liver.

brain from neurotoxins and is induced in response to certain neurotoxic insults. Toluene is known to cause a rapid increase in reactive oxygen species in the brain (33), and this oxidative stress could trigger increases in many proteins involved in protective functions .

Clozapine is one of the most useful drugs in the treatment

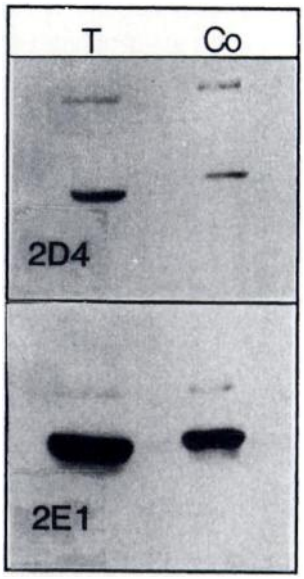


Fig. 8. Western blot analysis of the effect of toluene on P450s 2D4 and 2E1 in the liver. Microsomes from toluene-treated (*T*) and control (*Co*) rats were compared. On the blot probed with the 2D4 antibody, 40 pmol of P450 was loaded onto each lane; on the blot probed with the 2E1 antibody, 10 pmol of P450 was loaded.

of schizophrenia. Its use is not associated with tardive dyskinesia, a common toxicity associated with long term use of typical neuroleptic drugs. The mechanism by which clozapine exerts its beneficial effects, particularly on the negative symptoms of schizophrenia, is still unclear. It acts preferentially at the D_4 dopamine receptors and on serotonin receptors (18). Whether the induction of P4502D4 in the brain is related to the blockade of dopamine D_4 receptors remains to be established. It is clear from these studies that blockade of dopamine D_2 and serotonin receptors does not result in induction of this enzyme in the brain.

It has been suggested that identification of the major genes that are activated or repressed by long term treatment with clozapine will help to provide some insight into the mechanism of action of this important drug (18). Long term treatment with clozapine increased the overall content of P450 in the brain 4–6-fold. P4502D4 represents a small fraction of this P450, but its induction in neurons of the substantia nigra compacta and ventral tegmental area, regions related to the neuroleptic action of clozapine, suggests that induction should be considered a factor in the pharmacological action of clozapine. The remainder of the induced P450 must be identified and localized.

The role of P4502D4 in neurons is not yet known. There was no detectable metabolism of clozapine in the brains of control, clozapine-treated, or toluene-treated rats (data not shown). The 2D P450s are thought to have as their substrates some neurotoxin or neurotoxins acquired through the diet or endogenously produced by some metabolic pathway. Tetrahydroisoquinoline, a dopamine metabolite that can induce parkinsonian symptoms, is an example of such a compound (35). The function of the 2D enzymes would be elimination of these neurotoxins from the brain, and this could be their role in preventing Parkinson's disease. It has also been suggested that long term treatment with clozapine might induce genes that reduce neurotoxic processes and thus per-

mit or facilitate neural repair (18). We suggest that brain P450 should be strongly considered for such a role.

Of the CNS-active drugs used in this study, clozapine, mianserin, and sulpuride were inducers of P450 in the brain, but haloperidol was not. The forms of P450 induced and the cells that respond to these drugs by induction of P450 remain to be identified. Such information is necessary to clarify the issue of whether brain P450 participates in the therapeutic effects or in the CNS toxicity of these drugs.

Acknowledgments

The skillful assistance in immunohistochemical techniques of Anne Maatta is greatly appreciated.

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Send reprint requests to: Dr. Eva Hedlund, Department of Medical Nutrition, Karolinska Institutet, Huddinge University Hospital F60, NOVUM, 14186 Huddinge, Sweden.