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Brain CYP2E1 is induced by nicotine and ethanol in rat and is higher in smokers and alcoholics

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1 Ethanol and nicotine are commonly coabused drugs. Cytochrome *P*450 2E1 (CYP2E1) metabolizes ethanol and bioactivates tobacco-derived procarcinogens. Ethanol and nicotine can induce hepatic CYP2E1 and we hypothesized that both centrally active drugs could also induce CYP2E1 within the brain.

2 Male rats were treated with saline, ethanol (3.0 g kg^{-1} by gavage) or nicotine (1.0 mg kg^{-1} s.c.) for 7 days. Ethanol treatment significantly increased CYP2E1 in olfactory bulbs (1.7-fold), frontal cortex (2.0-fold), hippocampus (1.9-fold) and cerebellum (1.8-fold), while nicotine induced CYP2E1 in olfactory bulbs (2.3-fold), frontal cortex (3.0-fold), olfactory tubercle (3.1-fold), cerebellum (2.5-fold) and brainstem (2.0-fold). Immunocytochemical analysis revealed that the induction was cell-type specific.

3 Consistent with the increased CYP2E1 found in rat brain following drug treatments, brains from alcoholics and alcoholic smokers showed greater staining of granular cells of the dentate gyrus and the pyramidal cells of CA2 and CA3 hippocampal regions as well as of cerebellar Purkinje cells compared to nonalcoholic nonsmokers. Moreover, greater CYP2E1 immunoreactivity was observed in the frontal cortices in the alcoholic smokers in comparison to nonalcoholic nonsmokers and alcoholic nonsmokers.

4 To investigate if nicotine could contribute to the increased CYP2E1 observed in alcoholic smokers, we treated human neuroblastoma IMR-32 cells in culture and found significantly higher CYP2E1 immunostaining in nicotine-treated cells (0.1–10 nM).

5 CYP2E1 induction in the brain, by ethanol or nicotine, may influence the central effects of ethanol and the development of nervous tissue pathologies observed in alcoholics and smokers.
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Abbreviations: ADH, alcohol dehydrogenase; CYP, cytochrome *P*450; CYP2E1, cytochrome *P*450 2E1; CNS, central nervous system

Introduction

The members of the cytochrome *P*450 (CYP) enzyme family that metabolize drugs are found at high levels in the liver, but are also found in other tissues, including lung, kidney and brain (Hasler, 1999). Although CYP content in the central nervous system (CNS) is relatively low (Warner & Gustafsson, 1994), the observed regional and cellular localization of individual isozymes may lead to CYP levels in specific cell types that are considerably higher. Cytochrome *P*450 2E1 (CYP2E1) was found to be expressed in various rat brain regions (e.g. olfactory lobes) and cell types (e.g. astrocytes) (Hansson *et al.*, 1990; Howard *et al.*, 2000; Upadhya *et al.*, 2000). Moreover, this enzyme appears to be functional and inducible in CNS tissue (Montoliu *et al.*, 1995; Tindberg & Ingelman-Sundberg, 1996). Similarly, CYP2E1 expression has been detected in human brain regions, including the hippocampus, medulla and substantia nigra (Farin & Omiecinski, 1993).

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Endogenous substrates of CYP2E1 include arachidonic acid, fatty acids, gluconeogenic precursors and estrogenic metabolites (Lieber, 1999; Ohe *et al.*, 2000). Of interest, CYP2E1 was found to be expressed in dopaminergic neurons within rat substantia nigra (Hansson *et al.*, 1990) and may have a role in dopamine regulation (Nissbrandt *et al.*, 2001). These physiological roles may explain the similar catalytic properties and regulation of CYP2E1 across species, as observed between human and rat CYP2E1; hence, rat CYP2E1 is thought to be a good model for human CYP2E1 regulation (Lieber, 1999).

CYP2E1 also bioactivates several procarcinogens (e.g. tobacco-specific nitrosamines and benzene) and cytotoxins (e.g. carbon tetrachloride) to their reactive intermediates (Lieber, 1999). Moreover, CYP2E1 appears to have a role in the generation of reactive oxygen species, which initiate lipid peroxidation and DNA oxidation, thereby inducing cellular injury. CYP2E1 has been detected in prenatal human brain, suggesting a possible role in neurophysiology and/or neuroembryotoxicity (Brzezinski *et al.*, 1999).

There is evidence that oxidation of ethanol to acetaldehyde can occur in the brain (Zimatkin & Deitrich, 1997). CYP2E1 is also induced in liver by ethanol and has been proposed to contribute to the increased ethanol metabolism observed in alcoholics (Lieber, 1999). As mentioned, CYP2E1 is constitutively expressed in various brain regions (Hansson *et al.*, 1990; Howard *et al.*, 2000), whereas the major ethanol-metabolizing enzyme in liver, alcohol dehydrogenase I (Lowndes *et al.*, 1994) is not. Catalase has been proposed to contribute to ethanol metabolism in the brain (Zimatkin & Deitrich, 1997), but its contribution *in vivo* is questionable because its activity is dependent on H_2O_2 (Boveris *et al.*, 1972). Hence, induced CYP2E1 in brain is likely to have an important role in the metabolism of ethanol.

A higher percentage of alcoholics smoke (80–95%) compared to nonalcoholics (25–30%), and among nonalcoholics, smokers consume twice as much alcohol as nonsmokers (Batel *et al.*, 1995). Consistent with this coconsumption of ethanol and tobacco, nicotine administration to rats increases their self-administration of ethanol (Ericson *et al.*, 2000). The underlying mechanism(s) has not been elucidated, but nicotinic receptors or other receptor systems in the CNS or periphery may be involved in this interaction between nicotine and ethanol (Ericson *et al.*, 2000). In addition, the coabuse of these drugs may be partly because of tobacco smoke constituent(s) increasing the metabolic inactivation of ethanol, providing an impetus for increased ethanol consumption. We, and others, have found that chronic nicotine administration increases CYP2E1 protein and activity (Anandatheerthavarada *et al.*, 1993b; Howard *et al.*, 2001). The aims of this study were to determine the effects of nicotine and ethanol on CYP2E1 in rat brain, to examine brain CYP2E1 in human alcoholics and smokers, and to observe the effect of nicotine on CYP2E1 in a human neuronal cell line. Ethanol- or nicotine-mediated increases in brain CYP2E1 could influence local ethanol levels at sites of action in the CNS, susceptibility to toxic intermediates generated by CYP2E1, and metabolism and subsequent levels of endogenous CYP2E1 substrates.

Methods

Materials

Protease inhibitor cocktail tablets and Chemiluminescence Blotting Substrate™ were purchased from Roche Diagnostics (PQ, Canada). Recombinant virally expressed CYP2E1 lymphoblastoid Supersomes™ and Diachii's goat anti-rat CYP2E1 antibody were purchased from Gentest Corporation (MA, USA) and pyridine-induced rat liver microsomes were purchased from Oxford Biomedical Research, Inc. (ON, Canada). Protran™ nitrocellulose membranes were purchased from Schleicher & Schuell Inc. (NH, USA). Biotinylated goat anti-rabbit IgG antibody, biotinylated rabbit anti-goat antibody, avidin–biotin complex or ABC Elite kit and 3,3′-diaminobenzidine/hydrogen peroxide or DAB kit were purchased from Vector Laboratories Inc. (ON, Canada), and Neutravidin™-conjugated horseradish peroxidase was purchased from Pierce Chemical Company (IL, USA). Xylene and Permount was purchased from Fisher Scientific, ON, Canada. Protein assay kit and prestained molecular markers were purchased from Bio-Rad Laboratories (CA, U.S.A.). Ethanol, nicotine bitartrate and nicotine hemisulfate were purchased from Sigma-Aldrich Canada Ltd. (ON, Canada). Tissue culture flasks (80 cm^2) were purchased from Nunc, and 0.22 μM filters from Millipore. Dulbecco's modified Eagle's medium, glucose, Hanks balanced salts solution, fetal calf serum, gentamicin, poly-D-lysine were purchased from Gibco BRL (ON, Canada). IMR-32 cells were acquired from the American Type Culture Collection (VA, U.S.A.).

Animals

Adult, male Wistar rats (250–300 g; Charles River, P.Q., Canada) ($n = 4$ per treatment) were injected subcutaneously, once per day, for 7 days with saline or 1.0 mg of nicotine base per kilogram body weight, in the form of nicotine bitartrate in sterile saline adjusted to pH 7.4. Ethanol at 3.0 g kg^{-1} or saline was given once a day for 7 days by gavage to animals deprived of food for 2–4 h to facilitate constant absorption. Wistar rats were chosen for this study since they have been used to study induction of brain (Anandatheerthavarada *et al.*, 1993a; Warner & Gustafsson, 1994) and hepatic CYP2E1 (Howard *et al.*, 2001), ethanol-induced CNS damage (Montoliu *et al.*, 1994), and the effects of tobacco on ethanol metabolism (Marselos *et al.*, 1991). The dose of 3 g kg^{-1} ethanol was sufficient to produce observable CNS effects, including sedation and motor impairment (Le & Israel, 1994). The 1.0 mg kg^{-1} nicotine dose has been shown to mediate behavioral (i.e. hyperkinesia, tremors) and pharmacodynamic changes (i.e. receptor adaptation, dopamine release) in rodents that models the functional tolerance seen in humans (Howard *et al.*, 2001). This dose of nicotine induces hepatic CYP2E1 and brain CYP2B1 and produces plasma nicotine levels similar to those acquired by smoking 20 cigarettes in humans (Miksys *et al.*, 2000a; Howard *et al.*, 2001). At 4 h after the last drug administration, animals were killed by decapitation, and brains and livers were removed.

Brains were divided longitudinally; one-half of each brain was processed for immunocytochemistry and one-half for immunoblotting. The halves used for immunoblotting were dissected as described previously (Miksys *et al.*, 2000a) into seven regions, olfactory bulbs, frontal cortex, olfactory tubercle, hippocampus, striatum, cerebellum and brainstem. The regions examined were chosen to include areas examined by Anandatheerthavarada *et al.* (1993b) and areas that are involved in mediating the effects of ethanol and nicotine (Fadda & Rossetti, 1998; Miksys *et al.*, 2000a). Dissected brain regions were frozen immediately in liquid nitrogen and stored at −80°C. All experimental procedures described in this study were carried out in accordance with the guidelines for the care and use of laboratory animals and approved by the Animal Care Committee of the University of Toronto.

Studies with human brain

Human brain tissues were obtained from the University of Miami Brain Endowment Bank. The next of kin representing the deceased granted permission to retain brain tissue for research purposes. The protocols for obtaining post-mortem specimens have received Institutional Review Board authorization from the University of Miami School of Medicine. Post-mortem specimens were obtained during autopsy from drug-free control subjects, smokers and alcoholic subjects.

Medicolegal investigations of the deaths were conducted by forensic pathologists and brain tissues were examined using standard neuropathological procedures. The circumstances of death and toxicology data were reviewed prior to classification as an alcoholic case or as a control. All cases were evaluated for common drugs of abuse and alcohol and positive urine screens were confirmed by quantitative analysis of blood. Drug-free control subjects were selected from deaths because of accident or natural causes that came to post-mortem evaluation (Table 1). Information regarding alcohol consumption and cigarette use of each subject was obtained by structured interviews with next of kin. Smoking exposures were confirmed by measuring nicotine levels in whole blood or urine specimens. All of the alcoholic cases included in this study had confirmed histories of chronic alcohol abuse. Blood alcohol concentrations ranged from 0.09 to 0.38%. None of the cases had abused illicit or prescription drugs. Three human brain regions were chosen for immunocytochemical examination, frontal cortex, hippocampus and cerebellum, based in part on the findings from the animal studies.

Membrane preparation for immunoblotting

Rat brain regions and livers were homogenized manually in 100 mM Tris (pH 7.4) with 0.1 mM EDTA, 0.32 M sucrose, 0.1 mM dithiothreitol and protease inhibitor cocktail on ice. Homogenates were centrifuged twice at $3000 \times g$ for 3 min to remove cellular and nuclear debris, and the membrane fractions were prepared by $110{,}000 \times g$ centrifugation (Sorvall RC2-B Combi Plus Ultraspeed Centrifuge) of the supernatant fraction. The resulting membrane pellets were resuspended in 100 mM Tris (pH 7.4), 0.1 mM EDTA, 0.1 mM dithiothreitol, 1.15% w v^{-1} KCl, 20% v v^{-1} glycerol and stored until used at -80°C. Microsomes from untreated and pyridine-induced rat liver, as well as from lymphoblastoid expressed rat CYP2E1, were used as positive controls for the detection of brain CYP2E1 by immunoblotting. The protein content of each sample was assayed by the Bradford method using a Bio-Rad Protein Assay Kit (Bradford, 1976).

Immunoblotting

Untreated rat hippocampal (pooled) and liver membranes were serially diluted and used to construct standard curves in order to determine the linear range of detection for the immunoblotting assay. Membrane proteins, 50 μg of each test brain region, 2 μg of liver and 0.18 pmol of expressed rat CYP2E1 (used as positive control) were separated by SDS-PAGE (4% stacking and 8% separating gel) and then transferred overnight onto nitrocellulose membranes. The membranes were subsequently probed with a rabbit polyclonal antibody raised against rat CYP2E1 (generously donated by Magnus Ingelman-Sundberg, Karolinska Institutet, Stockholm) (Johansson *et al.*, 1988), diluted 1 : 3500 for 1.5 h at 20°C. Blots were then incubated with biotinylated goat anti-rabbit IgG for 1 h at 20°C, followed by Neutravidin™-conjugated horseradish peroxidase for 20 min at 20°C and developed using chemiluminescent detection. To control for nonspecific signals, blots were incubated either without primary anti-CYP2E1 antibody or with primary antibody that had been preadsorbed for 12 h with expressed CYP2E1 (1 μM).

Immunocytochemistry

Half rat brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 24 h, cryoprotected in 20% sucrose in phosphate buffer, and rapidly frozen in isopentane cooled on dry ice. Sections (16 μM thick) were cut on a freezing microtome, collected in six well plates containing PBS (10 mM sodium phosphate buffer, 0.9% sodium chloride, pH 7.4). Sections were washed three times in PBS and incubated free floating in PBS containing 4% Triton X-100, 5% normal horse serum, 2% bovine serum albumin and 1% skimmed milk for 2 h at room temperature. Sections were then incubated at 4°C for 4 days with the anti-rat CYP2E1 antibody (same as above) diluted 1 : 500 in PBS containing 2.5% Triton X-100, 1% normal horse serum and 1% bovine serum albumin. The antigen–antibody complex was visualized using a biotinylated rabbit anti-goat antibody followed by the

Table 1 Demographics, drug taking status of tissue donors

Case[a]	*Age*	*Gender*	*PMI*[b]	*Alcohol consumption*	*Smoking*[c]	*Cause of death*
C7	46	M	11	None	None	Myocardial infarction
C6	59	M	14	None	None	Myocardial infarction
Mean	52.5		12.5			
(s.d.)	(9.2)		(2.1)			
A9	58	F	21[d]	Chronic	None	Hepatic cirrhosis
A3	38	M	21	Chronic	Yes	Suicide (hanging)
A4	53	M	23	Chronic	1 pk/day	Cardiac disease
A6	48	M	18	Chronic	3 pk/day	Blunt trauma
A7	65	M	19	Chronic	2 pk/day	Cardiac disease
A8	52	F	22	Chronic	2–3 pk/day	Suicide (hanging)
A5	44	M	12	Chronic	Yes	Chronic alcoholism
Mean	50		19.2[d]			
(s.d.)	(9.2)		(4.0)			

No illicit drugs where detected in toxicology screens conducted at autopsy. SA3 was African American, all other cases were Caucasian.
[a]Cases categorized as C for nonsmoking nonalcoholics, A for nonsmoking alcoholics and SA for smoking alcoholics.
[b]PMI: post-mortem interval in hours.
[c]For smoking descriptors, 'yes' indicates unknown number of cigarette packs per day, pk: numbers of cigarette packs where a pack contains 20 cigarettes.
[d]PMIs of the alcoholics were lower than the nonalcoholics ($P < 0.05$, for comparison of nonalcoholic nonsmokers to alcoholic smokers).

avidin-biotin detection system and reaction with 3.3′-diaminobenzidine.

Immunocytochemical staining of human brain regions was performed using a similar protocol to that used for rat brain with some minor modifications as follows: brain tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and cryoprotected in 20% sucrose in buffer. Frozen sections were collected in 10 mM PBS, blocked for 1 h in 1% skim milk, 1% BSA, 2% normal horse serum (NHS) and 0.01% Triton X-100 in PBS, and incubated for 48–72 h at 4°C in goat anti-rat CYP2E1 antibody (diluted 1 : 2500 in 1% BSA, 2% NHS, 0.01% Triton X-100 in PBS). Endogenous peroxidase activity was quenched by incubating either rat or human brain sections for 5 min in 0.3% H_2O_2 in PBS after the secondary antibody. Negative control sections were treated identically except for the absence of primary antibody.

Cell culturing and immunocytochemistry

IMR-32 cells were cultured in 80 cm^2 tissue culture flasks at 37°C in 5% CO_2, 95% air and 100% humidity. Cells were grown in Dulbecco's modified Eagle's medium (4.5 $g\,l^{-1}$ glucose) in 45% Hanks buffered salt solution that contained 45% nonessential amino acids, 10% fetal calf serum and 50 $\mu g\,ml^{-1}$ gentamicin. Cells were passaged and 1×10^5 cells in 2 ml of medium were transferred into 10 cm well plates containing sterilized, poly-D-lysine-coated coverslips. After 24 h, the IMR-32 cells were treated with 0, 0.1, 1 or 10 nM of nicotine in medium for 48 h; nicotine solutions were made using a 2 M nicotine hemisulfate salt stock solution (pH = 7.4) sterilized using a 0.22 μM filter. These nicotine concentrations are consistent with plasma and brain nicotine levels that elicit behavioral responses in rodent models in pathways believed to underlie the development of nicotine dependence in smokers (Pratt *et al.*, 1983).

After removal of medium, the cells were washed in PBS and fixed using fresh 4% paraformaldehyde in PBS for 1 h at room temperature. For immunostaining, cells were washed in PBS and blocked in 1% Triton X-100, 1% BSA and 10% NHS in PBS 1 h prior to an overnight incubation at 4°C in goat anti-rat CYP2E1 antibody (diluted to 1 : 4000). Cells were then blocked in 1% BSA and 10% NHS in PBS for 1 h followed by 1 h in biotinylated rabbit anti-goat antiserum, processed using the avidin–biotin detection system and reacted with 3.3′-diaminobenzidine and hydrogen peroxide.

Optical density measurements and statistics

Immunoblots were analyzed and quantified with a digital imaging system from Imaging Research Inc., St Catherines, ON, Canada. Differences between treatment groups of animals were tested using unpaired Student's *t*-tests. All images, from both immunoblot and immunocytochemistry studies, were acquired by the imaging system under identical optical conditions and no further manipulations of image density were performed.

Results

CYP2E1 in untreated rat brain

An immunoblotting assay was developed to detect and quantify CYP2E1 in brain tissue where constitutive expression is low. The immunoreactive band from brain tissue comigrated with a band from untreated, or pyridine-treated, rat liver microsomes and from lymphoblastoid expressed rat CYP2E1 (Figure 1a). The CYP2E1-reactive signal detected in these tissues was linear from 30 to 90 μg of protein and specificity of the detection system was confirmed by incubating immunoblots without primary antibody or with preadsorbed primary antibody (Figure 1b). A CYP2E1-reactive signal could not be detected below 30 μg of untreated hippocampal membranes. Using this sensitive and quantitative immunoblotting assay, CYP2E1 was detected in the membranes of each of the brain regions examined (Figure 1c). There was variability among the different regions, with a 3.6-fold difference between the region with the highest (cerebellum) and the region with the lowest (striatum) amount of CYP2E1. As expected, the level of brain CYP2E1 was low compared to its hepatic content (Figure 1c).

Induction of CYP2E1 in rat brain by nicotine and ethanol

Treatment with a behaviorally relevant dose of ethanol (3 $g\,kg^{-1}$) for 7 days significantly increased brain CYP2E1 in a region-specific manner. Specifically, CYP2E1 expression was

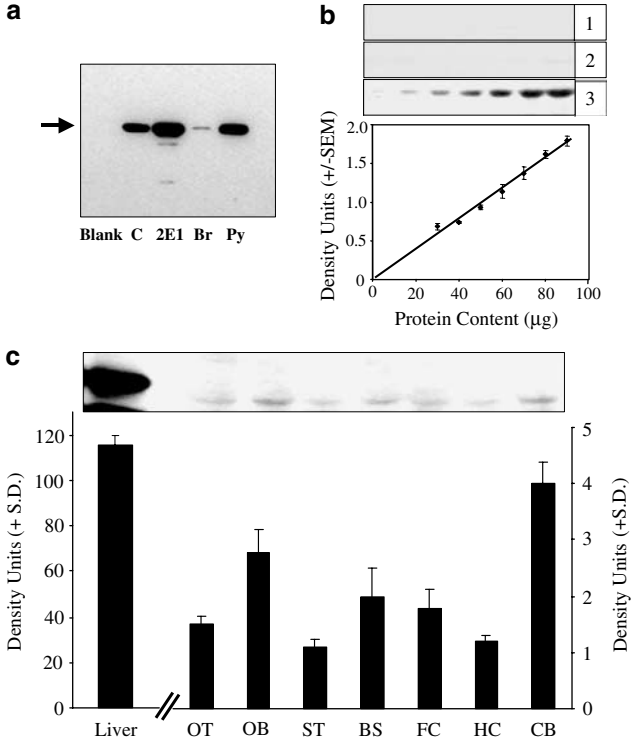

Figure 1 Immunoblotting of CYP2E1 in rat brain. (**a**) An immunoreactive band was detected in untreated rat hippocampal membranes (Br) which comigrated with immunoreactive bands from liver microsomes from rats that were untreated (C) or treated with pyridine (Py) and with expressed rat CYP2E1 (2E1). (**b**) Representative immunoblots incubated without primary antibody (1), with preadsorbed primary antibody (2) and with primary (3) antibody for construction of a standard curve of untreated rat hippocampal membrane (mean of three experiments, ±s.e.m.). (**c**) Rat CYP2E1 levels in 50 μg of membrane homogenates from olfactory tubercle (OT), olfactory bulb (OB), striatum (ST), brainstem (BS), frontal cortex (FC), hippocampus (HC) and cerebellum (CB) brain regions were compared to rat liver.

significantly increased in the olfactory bulb (1.7-fold), frontal cortex (2.0-fold), hippocampus (1.9-fold) and cerebellum (1.8-fold) (Figure 2a, b). A low dose of nicotine (1 mg kg^{-1}) for 7 days also increased CYP2E1 amounts in several regions of rat brain. Of note, there was a different pattern of regional increases in CYP2E1 following nicotine compared to ethanol treatments. The greatest elevations in CYP2E1 by nicotine were observed in frontal cortex (3.0-fold) and olfactory tubercle (3.1-fold) with smaller but significant increases in the olfactory bulbs (2.3-fold), cerebellum (2.5-fold) and brainstem (2.0-fold) (Figure 2c, d).

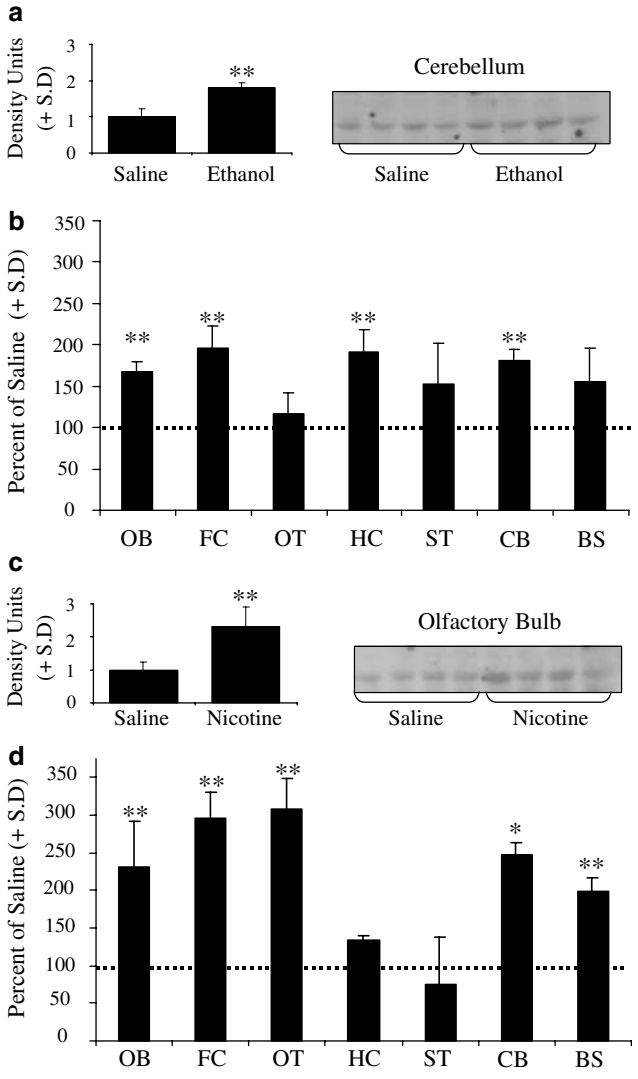

Figure 2 Chronic ethanol or nicotine-induced CYP2E1 in rat brain. (**a**) Ethanol increased CYP2E1 in rat cerebellum relative to saline-treated rats (right-hand panel is a representative immunoblot). (**b**) Ethanol-induced CYP2E1 levels in olfactory bulb (OB), frontal cortex (FC), hippocampus (HC) and cerebellum (CB) compared to saline treatment. (**c**) Nicotine increased CYP2E1 in rat olfactory bulb relative to saline-treated rats (right-hand panel is a representative immunoblot). (**d**) Nicotine increased CYP2E1 in olfactory bulb (OB), frontal cortex (FC), olfactory tubercle (OT), cerebellum (CB) and brainstem (BS) compared to saline treatment. ST represents the brain region striatum. Values are means +s.d. of four animals, each animal assayed five times. Significant differences from saline-treated animals are indicated by (*) for $P<0.05$ and (**) for $P<0.001$.

To ensure that the nicotine-mediated increase in CYP2E1 in olfactory tubercle (the largest change detected in CYP2E1) was in the linear range and therefore measured accurately by the assay, olfactory tubercle samples from nicotine-treated animals were diluted by 3.1-fold to the quantities estimated for saline-treated animals and then reanalyzed. Following this dilution, CYP2E1 protein amounts were the same for both treatments (data not shown), indicating that our system was not saturated at the higher amounts of CYP2E1 detection and we could use the assay to accurately determine CYP2E1 amounts over this range of induction.

Immunocytochemical analysis of CYP2E1 in rat brain

Very little CYP2E1 immunoreactivity was detected throughout rat brain sections from vehicle-treated rats (Figures 3 and 4, Table 2), which is consistent with the low quantities of CYP2E1 protein detected by immunoblotting (Figure 1c). In the cerebellum, the molecular and granular cell layers showed comparable staining and some glial cells were also stained in the subcortical white matter. Cerebellar Purkinje cells did not have detectable CYP2E1 immunoreactivity (Figures 3a and 4a). The olfactory bulbs stained lightly, with some glial staining in the anterior olfactory nucleus. Slight neuropilar staining was observed in the layers of the frontal cortex (Figure 4d) as well as glial cell immunoreactivity in the piriform cortex. The olfactory tubercle exhibited a moderate amount of staining. In the hippocampus, the pyramidal cell bodies and polymorphic layers of CA1 and CA2 regions also exhibited CYP2E1 immunoreactivity. There was also staining observed in the polymorphic layer of the dentate gyrus but not in the granular layer (Figure 3d).

Table 2 summarizes the alterations in CYP2E1 immunoreactivity for regions that were characterized by immunoblotting after treatment with either 3.0 g kg^{-1} ethanol or 1.0 mg kg^{-1} nicotine. Relative to control animals there was a general elevation in the overall staining of the frontal cortex in the ethanol group as well as the nicotine group, the latter is illustrated in Figure 4d, e. A similar increase in staining by ethanol and nicotine was seen in the piriform cortex and olfactory tubercle. Higher amounts of CYP2E1 immunoreactivity in ethanol and nicotine-treated animals were also observed in the striatum compared to their respective controls.

Administration of ethanol or nicotine led to an increase in CYP2E1 immunoreactivity in the granular and molecular cell layers of the cerebellum, while CYP2E1 in Purkinje cells remained undetectable post-treatment with either drug (Figures 3a, b and 4a, b). A increase in staining by ethanol treatment was also observed in the granular cell layer of the dentate gyrus (DG) (Figure 3d, e), with no change observed following nicotine. No immunoreactivity was observed with the omission of primary antibody followed by peroxidase quenching, while in the absence of peroxidase quenching, endogenous staining was only observed in the red blood cells (Figures 3c, f and 4c, f).

CYP2E1 immunocytochemical staining in human brain regions

CYP2E1 immunoreactivity was investigated in the three regions of human brain that had been studied in rats. A comparison of the demographics of the nine donors showed

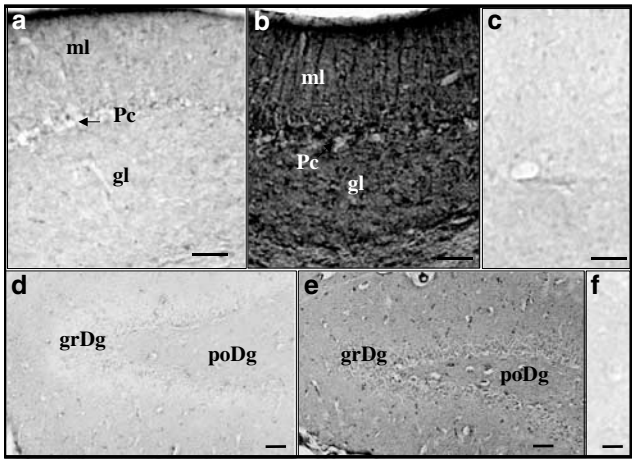

Figure 3 Low doses of ethanol increased CYP2E1 immunoreactivity in brain in a region- and cell type-specific manner. Immunocytochemical analysis demonstrated ethanol (**b**) increased CYP2E1 staining in the molecular layer (ml) and granular cell layer (gl) of the cerebellum relative to its saline control (**a**). The Purkinje cells (Pc) of the cerebellum showed negligible immunostaining. Hippocampal sections (coronal sections at Bregma 3.60 mm) from ethanol-treated rat (**e**) exhibited greater CYP2E1 immunoreactivity compared to saline-treated animal (**d**). Dentate gyrus' polmorphic layer (poDg) and granular layer (grDg) of the hippocampus are labeled. Control sections were processed without primary antibody (**c**, **f**). Bar: 100 μm.

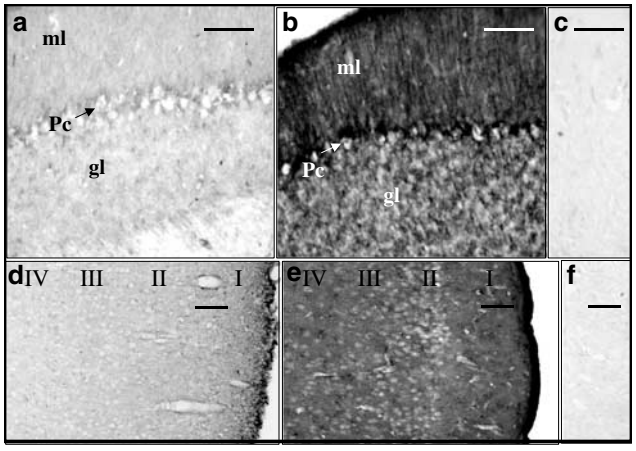

Figure 4 Increased CYP2E1 by low doses of nicotine was specific to region and cell-type. Higher CYP2E1 immunoreactivity was found in the molecular layer (ml) and granular cell layer (gl) of the cerebellum in nicotine-treated rats (**b**) compared to a vehicle-treated animal (a), but neglible CYP2E1 was detected in the Purkinje cells (Pc). Nicotine treatment moderately increased glial cell CYP2E1 immunostaining and slightly increased neuronal staining in the frontal cortex (e) compared to vehicle treatment (**d**). Layers I–IV of frontal cortex are indicated. Control sections were processed without primary antibody (**c**, **f**). Bar: 100 μm.

that apart from post-mortem interval there were no significant differences between the groups. 'Constitutive' CYP2E1 demonstrated distinct cellular staining in the nonalcoholic nonsmoker control group (Table 3). In the hippocampus, staining was also evident in the granular cells of the DG and in the pyramidal cells of the CA2 and CA3 layers. In the nonalcoholic nonsmoker group, little staining was apparent in

Table 2 Immunocytochemical analysis of brains from rats treated with ethanol (3 g/kg gavage) and nicotine (1 mg/kg s.c.) compared to saline controls ($n=4$ animals/group)

Brain region	*Saline*	*Ethanol*	*Nicotine*
Olfactory bulbs (OB)			
Olfactory nucleus	−/+	++	++
Lateral olfactory tract	−	−	−
Frontal cortex (FC)			
Neurons/glial cells (layers I, II, III, IV)	+	++	++
Olfactory tubercle (OT)	+	++	++
Piriform cortex	+	++	++
Striatum (ST)			
Caudate putamen	+	++	++
Corpus callosum	+	+	++
Hippocampus (HC)			
Dentate gyrus granule cells	−	−/+	−
Dentate gyrus polymorphic layer	+	++	+
CA1, 2 pyramidal cells	−/+	+++	−/+
CA1, 2 molecular/ polymorphic layer	+	+	+
Cerebellum (CB)			
Purkinje cells	−	−	−
Molecular layer	+	++	++
Granular cell layer	+	+++	++
White matter	−	−	+

Key: (+++) strong, (++) moderate, (+) weak, and (−) no staining.

the cerebellum with the exception of small neurons of the molecular layer and some granular cells that showed CYP2E1 immunoreactivity (Figure 5b). In the frontal cortex of nonalcoholic nonsmokers, there was no detectable CYP2E1 in layer I, but relatively intense staining was observed in small neurons in layer II and in the pyramidal neurons including their axons and dendrites in layers III and IV (Figure 6b). In addition, the small neurons and glial cells located in the white matter of the frontal cortex displayed slight immunoreactivity.

There was variation in cellular CYP immunostaining among the nonalcoholic nonsmokers, the alcoholic nonsmoker and the alcoholic smokers in all three brain regions examined. Specifically, in the cerebellar tissue from the alcoholic nonsmoker and alcoholic smokers, the Purkinje cell bodies and their projections into the molecular layer were intensely stained (Figure 5c, d), as compared to the negligible immunoreactivity observed in nonalcoholic nonsmokers (Figure 5b). The molecular layer of the cerebellum showed comparable degrees of immunoreactivity among the groups. There was slightly greater staining observed in the granular cell layer in the alcoholic smokers compared to other groups. The frontal cortices of the nonalcoholic nonsmokers and alcoholic nonsmoker displayed similar intensities of immunostaining in layers II–VI and in the white matter (Figure 6b, c), while in alcoholic smokers there was relatively greater immunoreactivity in pyramidal neuronal cell bodies and processes of layers III and V as well as greater cellular CYP2E1 immunostaining in the white matter (Figure 6d). In the hippocampus of the alcoholic nonsmoker and alcoholic smokers, the staining of granular cells of the DG and the pyramidal cells of CA2 and CA3 hippocampal regions was higher than in the nonalcoholic nonsmokers.

Table 3 CYP2E1 immunocytochemical staining of brain regions in humans

Brain region	*Non alcoholic nonsmoker (n=2)*	*Alcoholic nonsmoker (n=1)*	*Alcoholic smoker (n=6)*
Frontal cortex (FC)			
Layer I	–	–	–
Layer II–VI pyramidal neurons (cell bodies/processes)	+ +	+ +	+ + +
Glial cells	+ +	+ +	+ + +
White matter			
Scattered, small neurons and glial cells	+	+	+ +
Hippocampus (HC)			
Dentate gyrus granular cells	+	+ +	+ +
Pyramidal cells in CA2, 3	+	+ +	+ +
Cerebellum (CB)			
Molecular layer small neurons	+	+	+
Purkinje cells (cell bodies/processes)	–	+ + +	+ + +
Granular cell layer	+	+	+ +
White matter			
Scattered, small neurons and glial cells	–	+	+

Key: (+ + +) strong, (+ +) moderate, (+) weak, and (–) no staining.

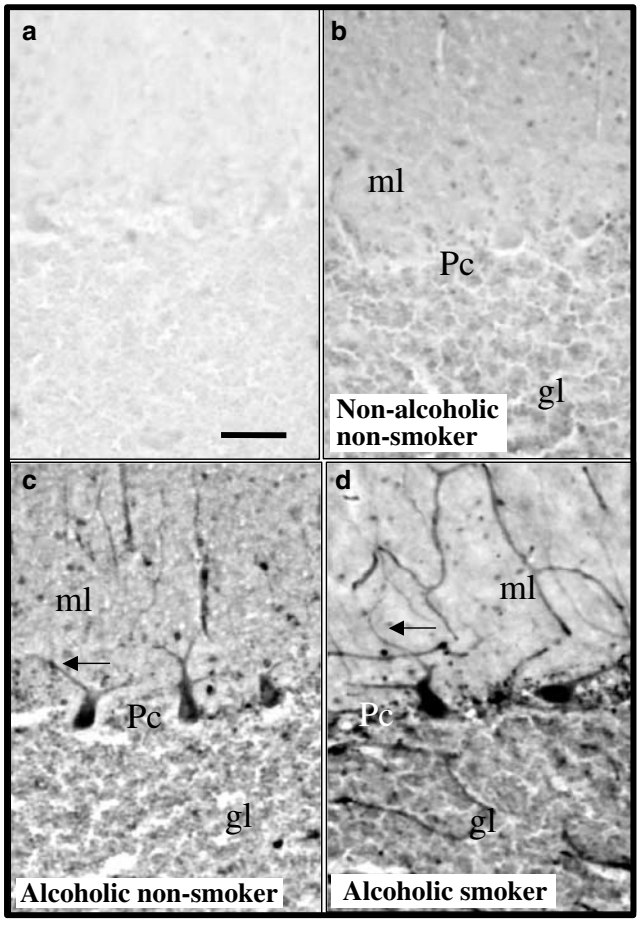

Figure 5 CYP2E1 immunoreactivity in human cerebellum. (**b**) Cerebellar layers of a nonalcoholic nonsmoker exhibited negligible staining of Purkinje cells (Pc), while Purkinje cells in cerebellum of an alcoholic nonsmoker (**c**) and an alcoholic smoker (**d**) exhibited very strong CYP2E1 immunoreactivity in Purkinje cell bodies and dendritic processes (arrows) in the molecular layer (ml). Granular cell layer (gl) of the alcoholic smoker showed greater CYP2E1 immunoreactivity than the nonalcoholic nonsmoker and alcoholic nonsmoker. (**a**) Cerebellar section processed without antibody. Bar: 100 μm.

Induction of CYP2E1 by nicotine in IMR-32 neuroblastoma cell line

As brains from human smokers who were not alcoholics were not available and in order to initiate investigations into the mechanism of regulation of human brain CYP2E1 by nicotine, studies in IMR-32 cell cultures were performed. IMR-32 cells are derived from a human neuroblastoma cell line that displays a neuronal phenotype; they are also of interest since these cells express $GABA_A$ and nicotinic acetylcholine receptors (Sapp & Yeh, 2000; Nelson *et al.*, 2001), which are targets for ethanol and nicotine. The ability of nicotine (0.1–10 nM) to induce CYP2E1 in IMR-32 cells was investigated by immunocytochemistry. These nicotine concentrations are within the range of plasma and arterial nicotine levels achieved in individuals who smoke or are on nicotine replacement therapy (Rose *et al.*, 1999). These nicotine concentrations are also consistent with plasma and brain nicotine levels that elicit behavioral responses in rodent models via pathways believed to underlie the development of nicotine dependence in smokers. We observed very low CYP2E1 cytoplasmic staining in untreated cells. We found very strong CYP2E1 immunoreactivity when the IMR-32 cells were treated with each of the three doses of nicotine compared to control cells (10 nM shown, Figure 7). These findings are the first observations of CYP2E1 in the IMR-32 cell line and suggest both the presence and inducibility of CYP2E1 by nicotine in these cells.

Discussion

Smokers appear to have greater ethanol metabolism rates and tobacco smoke has been shown to induce the ethanol-metabolizing enzyme CYP2E1 in animals and humans (Kopun & Propping, 1977; Jauhonen *et al.*, 1982; Lucas *et al.*, 1995; Villard *et al.*, 1998; Benowitz *et al.*, 1999); we have recently shown that nicotine can induce CYP2E1 in rat liver (Howard *et al.*, 2001). Therefore, we wanted to determine if nicotine, the addictive drug that maintains cigarette smoking, alters CYP2E1 in rat brain; this could contribute to the coabuse of ethanol and tobacco. Brain regions studied included those

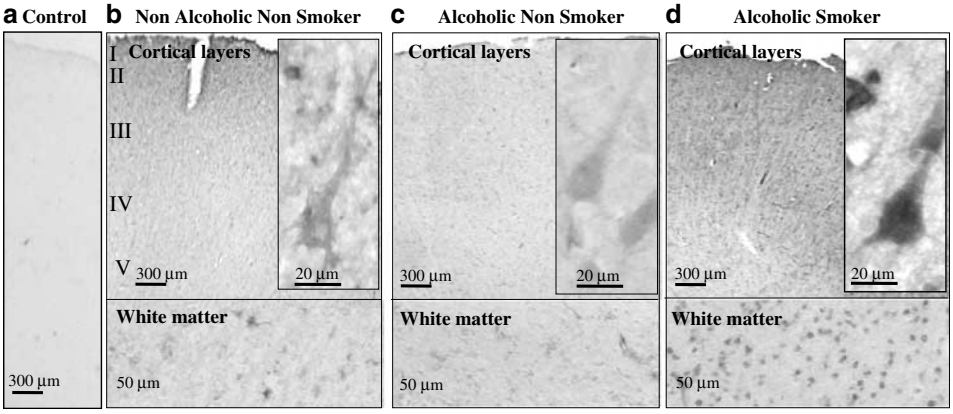

Figure 6 CYP2E1 immunoreactivity in human frontal cortex. Frontal cortex samples were obtained from the superior and middle frontal gyri, Brodmann areas 9 and 10. (**a**) Frontal cortical sections processed without primary antibody. (**b**) Frontal cortical sections of a nonalcoholic nonsmoker exhibited moderate staining of the pyramidal neurons cells in cortical layers II–V (enlarged in inset) and some staining of astrocytes in the white matter, which was similar to staining patterns observed in an alcoholic nonsmoker (**c**). (**d**) Sections from an alcoholic smoker showed very intense staining of the pyramidal neurons in the frontal cortical layers and intense cellular staining the in white matter.

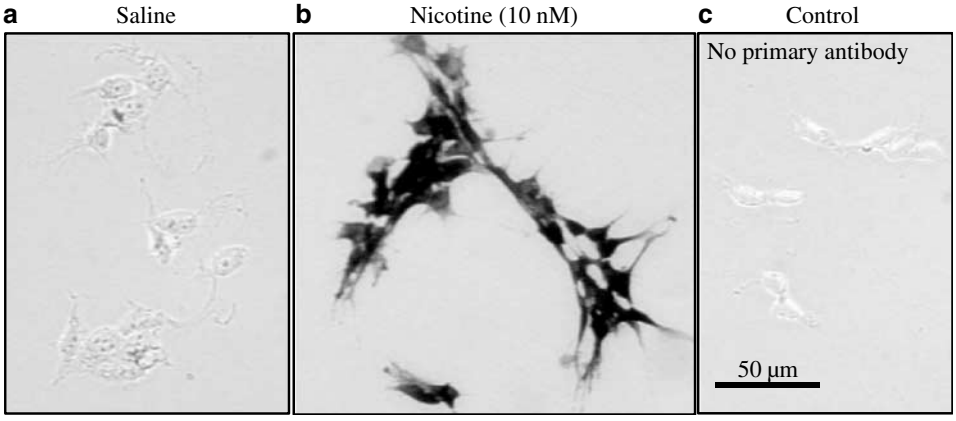

Figure 7 Immunocytochemical analysis demonstrated that nicotine (0.1–10 nM) increased CYP2E1 immunoreactivity in IMR-32 neuroblastoma cells. Representative images of cells treated with (**a**) saline or with (**b**) nicotine (10 nM) for 48 h; no immunoreactivity was detected when the primary antibody was omitted (**c**).

known to contain nicotinic receptors, ethanol targets and sites of ethanol-induced neuronal changes (Fadda & Rossetti, 1998; Tyndale & Tomkins, 1999; Le *et al.*, 2000; Miksys *et al.*, 2000a) along with regions examined in previous CYP2E1 studies (Anandatheerthavarada *et al.*, 1993b). We found that low, chronic doses of nicotine, like ethanol, can induce CYP2E1 in a region- and cell-specific pattern within the CNS.

In untreated animals, low quantities of CYP2E1 were present predominantly in evolutionarily older areas of the allocortex, such as the olfactory bulbs, olfactory cortex, hippocampus, cerebellum and brainstem. For example, hippocampal CYP2E1 expression was consistent with the amount of CYP2E1-mediated chlorzoxazone 6-hydroxylation determined previously in hippocampal homogenates (Tindberg & Ingelman-Sundberg, 1996). In addition, the cerebellum and olfactory bulbs contained the highest CYP2E1 amount, which is supported by previous findings of CYP2E1 activity and mRNA in brain (Upadhya *et al.*, 2000). Our immunoblotting data may differ slightly from previous investigations since we used whole-membrane preparations rather than microsomal preparations as CYP2E1 has been detected in the membranes of mitochrondria, lysosomes, peroxisomes, Golgi bodies, as well as the outer plasma membrane surface (Walther *et al.*, 1986; Neve & Ingelman-Sundberg, 2000).

A novel finding of this study is that low levels of nicotine *in vivo* increased CYP2E1 in specific rat brain regions and cell types and increased human CYP2E1 in cultured neurons. This is consistent with previous higher dose studies where nicotine increased brain enzymatic activities attributed to CYP2E1 (Anandatheerthavarada *et al.*, 1993b). Moreover, our immunocytochemical analysis revealed that induction occurred in cell types that we and others have found to constitutively express CYP2E1 (Hansson *et al.*, 1990). Nicotine treatment has been shown to attenuate some central effects of ethanol (Ericson *et al.*, 2000) and was suggested to lead to lower blood ethanol levels (Chen *et al.*, 2001). Our data suggest that region- and cell-specific CYP2E1 induction by nicotine could alter local concentrations of ethanol near receptor targets. Moreover, brain tissue is rich in unsaturated fatty acid side chains and low in antioxidant defences (e.g. glutathione), and may be

particularly susceptible to oxidative stress generated by nicotine induced-CYP2E1 (Montoliu *et al.*, 1994). Notably, ethanol and nicotine coadministration synergistically increased the generation of oxidative stress in rats (Ashakumary & Vijayammal, 1996). Moreover, increased amount of CYP2E1 by *in vivo* cigarette smoke exposure was implicated in mediating DNA strand breaks, which along with CYP2E1's ability to bioactivate procarcinogens (e.g. *N*-dimethylnitrosamine), may increase the risk for cancer (Toneatto *et al.*, 1995; Villard *et al.*, 1998; Lieber, 1999).

A behaviorally relevant dose of ethanol increased CYP2E1 in rat brain (1.7–2.0-fold) in a region- and cell-specific manner. Induction by ethanol in frontal cortex, olfactory bulb, hippocampus and cerebellum is consistent with most previous findings (Warner & Gustafsson, 1994; Tindberg & Ingelman-Sundberg, 1996; Upadhya *et al.*, 2000). However, one study did not find that chronic ethanol-treatment induced cerebellar CYP2E1 (Upadhya *et al.*, 2000), which may be because of differences in drug dosing and experimental assessments (Roberts *et al.*, 1994). Of note, CYP2E1 immunoreactivity in the olfactory tubercle was increased in the ethanol-treated rat group while this was not detected by immunoblotting of the whole region, suggesting that techniques that examine cellular distributions may be more sensitive. This is also evident with our immunocytochemical analysis of rat striatal CYP2E1, where we observed increased immunoreactivity in both ethanol and nicotine treatment groups which was not detected by immunoblotting. In addition, the pattern of striatal CYP2E1 immunoreactivity was altered in the nicotine group compared to the saline controls (data not shown), indicating that the distribution of CYP2E1 in particular brain regions may be altered by nicotine. Our data suggest ethanol and nicotine increase CYP2E1 where ethanol is thought to exert its pharmacodynamic effects as well as in regions known to undergo pathological changes during chronic ethanol consumption (Fadda & Rossetti, 1998).

Subsequently, we examined CYP2E1 immunoreactivity in the brains of alcoholics and alcoholic tobacco smokers. In the regions studied we observed higher amounts of CYP2E1 immunoreactivity in the cerebella, hippocampi and frontal cortices of alcoholic smokers compared to nonalcoholic nonsmokers (Figures 5 and 6, Table 3). We also found a greater amount of hippocampal and cerebellar CYP2E1 immunostaining between one alcoholic nonsmoker and the nonalcoholic nonsmokers, while there was a lower amount of frontal cortical CYP2E1 immunostaining in this alcoholic nonsmoker compared to the alcoholic smokers. However, these observations are based on small sample numbers and will need to be confirmed in a larger study. Chronic ethanol intake leads to cortical neuronal loss from the superior frontal cortex and hippocampus as well as a reduction in the number of cerebellar Purkinje cells (Fadda & Rossetti, 1998); higher amounts of CYP2E1 were observed in the same cell types that are injured by chronic ethanol consumption. In addition, we found slightly greater CYP2E1 immunoreactivity in pyramidal neurons, small neurons and glial cells of the frontal cortex in alcoholics who smoked compared to those who did not smoke. It should be noted that the mean post-mortem time interval was marginally shorter for the nonalcoholic cases compared to the alcoholic cases indicating the possible greater experimental loss of CYP2E1 in the alcoholics compared to the nonalcoholics in this study. This may have led to an underestimation of *in vivo* CYP2E1 expression after long-term abuse of alcohol and tobacco.

This study as well as others have noted species differences in CYP immunoreactivity in the cerebellum, depending on the particular isozyme. Human cerebellar Purkinje cells showed moderate to intense immunostaining of CYP2E1 and CYP2B6 (authors' unpublished observations), but this was not observed in rats (Hansson *et al.*, 1990; Miksys *et al.*, 2000a). Conversely, CYP2D expression in cerebellum of humans and rats appears to be more similar (Miksys *et al.*, 2000b; Miksys *et al.*, 2002).

Owing to unknown differences between human samples in both genetics and environmental exposures, we can only speculate that CYP2E1 expression was increased to a greater extent in alcoholics who smoke relative to alcoholic nonsmokers owing to tobacco smoking; this warrants further study. We have also found greater CYP2D6 immunostaining in alcoholics compared to nonalcoholics in the brain regions studied here, consistent with animal studies (Miksys *et al.*, 2002). Since CYP2D6 metabolizes neuroactive endogenous compounds and drugs, the possible greater amounts of CYP2D6 in alcoholics compared to nonalcoholics, which also appears to the case for CYP2E1, may contribute to the neurophysiological effects of ethanol. The increase in hippocampal $GABA_A$ receptor mRNA and CYP2E1 observed in our ethanol self-administration rat model may also occur in human alcoholics (Lewohl *et al.*, 1997) and represent a neuroplastic response to ethanol exposure and/or a genetic risk factor for alcohol consumption. This initial evaluation of CYP2E1 in human brain suggests that in alcoholic nonsmokers and smokers, elevations of this enzyme could lead to increased drug metabolism within the brain as well increased risk for CYP2E1-mediated neurotoxicity.

Unlike in animal models, detailed study of the potential induction of brain CYP2E1 by nicotine in humans is not feasible, therefore, we have begun studies investigating the effect of nicotine in human neural cells in culture using the human neuroblastoma IMR-32 cell line. We found this cell line expresses CYP2E1 at very low amounts, however, cells treated for 48 h with low nicotine concentrations showed significant CYP2E1 induction; the induction was maximal even at the lowest nicotine concentration tested. Low doses of ethanol are thought to induce hepatic CYP2E1 via protein stabilization (Zhukov & Ingelman-Sundberg, 1999). A recent study found that chronic ethanol administration increased CYP2E1 protein in rat brain, but did not alter CYP2E1 mRNA (Upadhya *et al.*, 2000). Similarly, we found a significant increase in hepatic CYP2E1 protein by ethanol or nicotine in the rats used in this study, but no change in the mRNA amounts (Howard *et al.*, 2001). Since we have determined that nicotine itself is able to increase CYP2E1 in human neuronal cells, we now have a tool for investigating the mechanism of nicotine's regulation of CYP2E1 in human CNS neurons.

Collectively, our results suggest that like ethanol, low doses of nicotine induce CYP2E1 in rat brain regions to a similar extent to rat liver (Howard *et al.*, 2001). We also found some evidence of elevated CYP2E1 in certain brain regions of human alcoholic nonsmokers and smokers compared to nonalcoholic nonsmokers. Overall, our study increases our understanding of the agents that regulate CYP2E1 in the brain which may, in turn, influence interindividual variation in the abuse of psychoactive drugs, the actions of protoxins, as well as levels of endogenous CYP2E1 substrates.

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