Differential expression of human cytochrome P450 enzymes from the CYP3A subfamily in the brains of alcoholics and drug free controls

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Non-standard abbreviations used are:

P450, Cytochrome P450, heme-thiolate protein P450; BSA, bovine serum albumin; OD, optical density; PBS, Phosphate Buffered Saline; PBST, Phosphate Buffered Saline containing 0.05 % (v/v) Tween-20; PKA, Protein kinase A; PKC, Protein kinase C.
Abstract

Cytochrome P450 (P450, CYP) enzymes are responsible for the metabolism of most commonly used drugs. Amongst these enzymes, CYP3A forms mediate the clearance of around 40-50% of drugs and may also play roles in the biotransformation of endogenous compounds. CYP3A forms are expressed both in the liver and extrahepatically. However little is known about the expression of CYP3A proteins in specific regions of the human brain. In this study, form-selective antibodies raised to CYP3A4 and CYP3A5 were utilized to characterize the expression of these forms in the human brain. Both CYP3A4 and CYP3A5 immunoreactivity was found to varying extents in the microsomal fractions of cortex, hippocampus, basal ganglia, amygdala and cerebellum. However, only CYP3A4 expression was observed in the mitochondrial fractions of these brain regions. N-terminal sequencing confirmed the principal antigen detected by the anti-CYP3A4 antibody in cortical microsomes to be CYP3A4. Immunohistochemical analysis revealed that CYP3A4 and CYP3A5 expression was primarily localized in the soma and axonal hillock of neurons and varied according to cell type and cell layer within brain regions. Finally, analysis of the frontal cortex of chronic alcohol abusers revealed elevated expression of CYP3A4 in microsomal but not mitochondrial fractions; CYP3A5 expression was unchanged. The site-specific expression of CYP3A4 and CYP3A5 in the human brain may have implications for the role of these enzymes in both normal brain physiology and the response to drugs.
Introduction

Cytochrome P450 (CYP, P450) enzymes (EC 1.14.13 and 1.14.14 groups) are monooxygenases that catalyze the oxidative metabolism of a great variety of xenobiotic and endogenous chemicals such as drugs, environmental contaminants, steroids and fatty acids. P450s of families CYP1-CYP3 show particularly wide and overlapping substrate ranges, a property which enables them to mediate approximately 75% of the metabolic clearance of drugs in humans (Wienkers and Heath, 2005). Most of the drug-metabolizing forms are expressed at high levels in the liver but many also show a widespread pattern of extrahepatic distribution (Guengerich, 2005). Several members of families CYP1-CYP3 have been reported in human brain (reviewed in (Meyer et al., 2007; Dutheil et al., 2010)) and the importance of these P450s in the metabolism of endogenous compounds in the brain and their potential role in normal neurological function and disease has been highlighted recently (Strobel et al., 2001; Haining and Nichols-Haining, 2007; Meyer et al., 2007; Niwa et al., 2009).

CYP3A4 is widely accepted to be the most important of the drug-metabolizing P450s in the liver, in both qualitative and quantitative terms, since it is expressed at high levels, metabolizes around 40-50% of all known therapeutic compounds (Krishna and Shekar, 2005) and deals with chemicals of diverse size and structure (Guengerich, 2005). Three additional CYP3A forms exist in humans: CYP3A5, CYP3A7 and CYP3A43. These P450s have been less well characterized but are usually expressed at lower concentrations (if at all) in the liver, show altered tissue specific distribution, and catalyze overlapping but different and generally more restricted ranges of metabolic activities compared to CYP3A4 (Guengerich, 2005; Daly, 2006; Stevens, 2006; Niwa et al., 2008). Differential expression of individual P450s and their inducibility by
exogenous compounds may influence the disposition of drugs within the brain and point to possible physiological roles of P450s in this tissue.

CYP3As have been shown to be expressed in the human brain at the mRNA level (Farin and Omiecinski, 1993; Murray et al., 1995; McFadyen et al., 1998; Kirches et al., 1999; Nishimura et al., 2003; Lamba et al., 2004; Agarwal et al., 2008; Dutheil et al., 2009). CYP3A-related mRNA has been detected in cortex, basal ganglia, hippocampus, midbrain and cerebellum, where expression seems to be mainly localized in neurons (Farin and Omiecinski, 1993; Pai et al., 2002; Agarwal et al., 2008). CYP3A4 mRNA has been specifically detected in cortex, cerebellum, basal ganglia, thalamus, medulla, dura mater, paracentral gyrus and corpus callosum (McFadyen et al., 1998; Lamba et al., 2004; Agarwal et al., 2008; Dutheil et al., 2009). CYP3A5 appears to be more widely distributed at the mRNA level, having been detected in dura mater, cortex, cerebellum, basal ganglia (nucleus accumbens and putamen), midbrain (red nucleus), pons, hippocampus, medulla, post- and para-central gyrus, corpus callosum, insula (McFadyen et al., 1998; Agarwal et al., 2008; Dutheil et al., 2009) and anterior pituitary (Murray et al., 1995). Finally, CYP3A43 mRNA has also been reported in human brain, where it seems to localize to the cortex, cerebellum, basal ganglia (nucleus accumbens), midbrain (reticular neurons and red nucleus), pons, hippocampus, dentate gyrus, medulla, spinal cord, and dura mater (Agarwal et al., 2008; Dutheil et al., 2009). Interestingly, its expression in human brain seems to be higher than in liver from the same individual, and equal to or higher than brain CYP3A4 (Agarwal et al., 2008).

Studies have also suggested that brain CYP3A forms are capable of metabolizing xenobiotic compounds (Voirol et al., 2000; Pai et al., 2002; Agarwal et al., 2008; Ghosh et al., 2010); however data on specific protein expression is limited (Ghosh et al., 2011). Detection of
individual CYP3A proteins in the brain has been hindered by a lack of antibodies directed to specific subfamily members, as well as limited access to adequate amounts of human brain tissue. The objective of the current study was to elucidate the pattern of CYP3A4 and CYP3A5 protein expression in five regions of the human brain (frontal cortex, hippocampus, basal ganglia, amygdala and cerebellum) and to assess the relative expression of CYP3A4 and CYP3A5 in alcoholic subjects.
Materials and Methods

Materials

Polyvinylidene Fluoride (PVDF) membrane and BioTrace™ NT nitrocellulose membranes were obtained from Pall Corporation (East Hills, New York). A number of commercially available antibodies were used in this study. Mouse anti-human-α-tubulin monoclonal primary antibody was purchased from Sigma (St. Louis, MO). The secondary antibodies used for human brain tissue immunoblots were AlexaFluor 680-labeled goat anti-rabbit IgG antibody (Invitrogen, Carlsbad California) and IRDye 800-labeled donkey anti-mouse IgG antibody (Rockland Immunochemicals, Gilbertsville, PA). The Odyssey infrared system was utilized for immunoblot imaging and analysis (LI-COR, Lincoln, NE). An AlexaFluor 488-labeled goat anti-rabbit IgG secondary antibody was used for immunohistochemistry (Invitrogen, Carlsbad California). Fluorescence mounting medium (DAKO, Glostrup, Denmark) was used to maintain fluorophore stability.

Antibody production

CYP3A4 and 3A5 specific antibodies were generated utilizing bacterially expressed recombinant proteins, expressed and purified as previously described (Gillam et al., 1993; Gillam et al., 1995). Pre-immune serum (20 ml) was collected from two 12-week old New Zealand white rabbits then purified, recombinant CYP3A4 or CYP3A5 (100 μg) was injected into each animal. Three subsequent boosters of 50 μg each were administered fortnightly thereafter. Animals were exsanguinated 11 weeks after initial immunization and serum was prepared as previously described (Depaz et al., 2003). IgG fractions were then prepared by sodium sulfate precipitation
(Harlow and Lane, 1999), followed by de-salting on a PD-10 desalting column and elution in phosphate buffered saline (PBS) containing 0.02% (w/v) sodium azide. Antibody aliquots (~200 μl) were snap frozen in liquid nitrogen and stored at -80°C.

To further enhance specificity, antibodies were isolated from immunoblots as previously described (Worrall et al., 1993). Briefly, bacterial membrane fractions containing 1800 pmol of recombinant CYP3A4 or 1400 pmol of recombinant CYP3A5 were electrophoresed on SDS-polyacrylamide gels (4% stacking gel and 10% resolving gel) then transferred onto a PVDF membrane (Towbin et al., 1979). Membranes were blocked with 1.35% (w/v) fish gelatin in phosphate buffered saline (PBS) for 1 hour at room temperature, then incubated with primary antibody overnight at 4°C at a dilution of 1:1000 in 1.35% (w/v) fish gelatin in PBS. Membranes were then washed and incubated with secondary Alexa 680-labeled goat anti-rabbit antibody (1:20,000) for 30 minutes at 25°C, before the washing and visualization on the Odyssey imaging system. The specific CYP3A4 or CYP3A5 band was excised from the membrane and the primary antibody was eluted with 2 mL elution buffer (200 mM glycine, 0.2% (w/v) gelatin, pH 2.8) for 2 minutes at 37°C. The eluate was neutralized with an equal volume of 1 M Tris base (no pH adjustment) and the resulting affinity-purified antibody preparation was used immediately in further immunoblotting experiments. Affinity-purified antibodies raised against CYP3A4 or CYP3A5 were tested for specificity to all four recombinant human CYP3A proteins (CYP3A4, CYP3A5, CYP3A7 and CYP3A43) expressed in bacterial membrane fractions. Equimolar amounts of each recombinant enzyme (0.5 pmol of spectrally detectable P450) were subjected to SDS-PAGE, and transferred to PVDF membranes as described above. Blots were incubated with affinity-purified primary anti-P450 antibodies at a dilution of 1:10 in 1.35% (w/v) fish gelatin in PBS and incubated on membranes overnight at 4°C. Incubation with AlexaFluor 680-labeled
goat anti-rabbit IgG secondary antibody and fluorescence detection was performed as described above. For each antibody, the cross-reactivity for individual P450 forms was expressed as the ratio of integrated intensity (I.I.) for the P450 in question to that of the target CYP3A (I.I._CYP/I.I._CYP3A4/5).

*Preparation of mitochondrial and microsomal protein fractions*

Frozen human brain samples were obtained from the New South Wales Tissue Resource Centre. For the first part of the study in which the sub-cellular localization of CYP3A4 and CYP3A5 was examined in five brain regions (frontal cortex (Brodman area 9 – BA9), anterior hippocampus, basal ganglia, amygdala and cerebellum), samples from three different male subjects (a total of 15 tissue samples) were provided as dissected sections. The samples were taken from the right hemisphere of alcohol- and illicit drug-free patients. The histological appearance was reported as normal at both the macroscopic and microscopic level. Details of patient histories are provided in Table 1. The samples utilized to compare the expression of P450s in the alcoholic and non-alcoholic brain were obtained from 12 different male subjects, the details of which are listed in Table 2. Frozen brain tissue samples (~500 mg) were placed into 5 mL of ice-cold homogenization solution (1.14% w/v KCl and 10 mM EDTA, pH 7.5, with 0.1 mM PMSF, 0.1 mM dithiothreitol, and 0.1 mM butylated hydroxytoluene added immediately prior to homogenization). Samples were manually crushed with a Teflon homogenizer for no more than 45 seconds (approximately 10-12 repetitions) then kept on ice prior to centrifugation at 3,000 g for 5 minutes at 4°C. The supernatant (S3000 fraction) was then centrifuged at 10,000 g for 30 minutes at 4°C. The resulting supernatant (S10000 fraction) was separated from the pellet and centrifuged at 110,000 g for 60 minutes at 4°C. Mitochondrial (10,000 g) and microsomal
(110,000 g) pellets were then resuspended in a volume of ice-cold buffer (250 mM sucrose and 10 mM EDTA, pH 7.5) equivalent to 3.2 and 1.6 fold their wet weight respectively. Aliquots (25 μL) of each fraction were snap frozen in liquid nitrogen and stored at -80°C.

**Immunoblot analysis**

Three control brain samples (Table 1) were used for immunoblot analysis of CYP3A4 and CYP3A5 expression in different brain regions and subcellular fractions. Samples (20 μg protein per lane) were separated on SDS-PAGE and subjected to immunoblotting with affinity-purified anti-CYP3A antibodies as described in section 2.2. Tissue samples were obtained from 12 different subjects for the analysis of CYP3A4 and CYP3A5 expression in the frontal cortex of alcoholics and non-alcoholics (Table 2). Blots were prepared as described above and incubated with affinity-purified primary anti-P450 antibodies and AlexaFluor 680-labeled goat anti-rabbit IgG secondary antibody before analysis using the Odyssey system. Mouse anti-human-α-tubulin monoclonal primary antibody (used at a dilution of 1:20,000) was utilized as a loading control in these experiments and detected using an IRDye 800-labeled donkey anti-mouse IgG secondary antibody (1:20,000; Rockland Immunochemicals, Gilbertsville, PA).

**Fluorescent immunohistochemistry of paraffin embedded sections**

Slide-mounted 4 μm brain sections were first cleared of paraffin as previously described (Depaz et al., 2003), then immersed in 10 mM sodium citrate buffer, pH 6.0 containing 0.05 % (v/v) Tween-20, and heated to 97°C for 30 minutes. Sections were left to cool for 30 minutes and
washed with PBS containing 0.05% (v/v) Tween-20 (PBST). Next, slides were blocked with 3% (w/v) bovine serum albumin (BSA) in PBS for 1 hour at room temperature before incubation with primary rabbit polyclonal P450 antibody (1:500) overnight at 4°C. Slides were then washed with 1% (w/v) BSA in PBS and incubated with secondary AlexaFluor 488-labeled goat anti-rabbit IgG antibody (1:1000) for 2 hours at room temperature in the dark. Finally, sections were washed with PBST and rinsed in PBS before being treated with 1% (w/v) Sudan Black for 5 minutes to quench lipofuscin autofluorescence. Sudan Black was washed off with 70% methanol and coverslips were applied to samples with fluorescence mounting medium (DAKO, Glostrup, Denmark). Slides were stored at -20°C in the dark until analysis. Images were visualized using a confocal fluorescent microscope and the cognate digital imaging software (Zeiss, Gottingen, Germany).

**N-terminal sequencing**

Samples for N-terminal sequencing were generated using the antibodies prepared as described in section 2.2. Briefly, anti-CYP3A4 antibodies were bound to Pierce Amino Link Plus affinity purification columns (Thermo Fisher Scientific, Rockford, IL, USA) as per the manufacturer’s instructions. Cortical cytosol samples (40 ug total protein) pooled from the three patients described above (Table 1) were incubated on the CYP3A4 antibody columns as per the manufacturer’s instructions overnight at 4°C. Target proteins were eluted and samples were concentrated down to <50 μL using a cryo-vacuum and semi-dry blotted onto PVDF membrane. N-terminal sequencing was performed by Edman degradation using an Applied Biosystems Procise 492 cLC automated sequencer.
Statistical analysis of immunoblots

Immunoblot images were scanned using the Odyssey imaging system, as per the manufacturer’s instructions. For quantitation of the alcoholic frontal cortex immunoblots, individual sample integrated intensities (I.I.) were corrected against α-tubulin immunoreactivity (I.I. of target protein/I.I. α-tubulin) and the corrected measurements were used for statistical analysis. The linearity range of the α-tubulin signal was assessed in immunoblots with increasing amounts (0 to 30 μg) of frontal cortex microsomal protein from one control sample. Data were subjected to paired Student-Newman-Keuls t-test analysis with a confidence interval (CI) of 95%. Data are expressed as mean ± SEM.
Results

Characterization of antibody selectivity

When tested for specificity against recombinant CYP3A enzymes, affinity-purified anti-CYP3A4 and 3A5 antibodies showed cross-reactivity with other forms to different extents (Supplementary Figure 1). For the antibody raised against CYP3A4, the cross-reactivity for each individual form was expressed as the ratio of integrated intensity for the P450 in question to that of CYP3A4 (I.I.CYP/I.I.CYP3A4), and was 0.51 and 0.93 for CYP3A5 and CYP3A7 respectively. For the antibody raised against CYP3A5, the cross-reactivity was expressed as the ratio I.I.CYP/I.I.CYP3A5, and was 0.51 and 0.81 for CYP3A4 and CYP3A7 respectively. No cross-reactivity was observed with CYP3A43 for either antibody under the high stringency conditions used, where blots were intentionally overloaded with recombinant antigens.

We cannot exclude the possibility that in immunoblotting and immunocytochemistry with brain samples the antibody raised against CYP3A4 is also detecting CYP3A7 and possibly CYP3A5, and that the antibody raised against CYP3A5 is also detecting CYP3A7 and possibly CYP3A4. However, two factors are important to note in the interpretation of these data. Firstly, expression of CYP3A7 and CYP3A43 holoenzyme in *E. coli* is poor relative to that of CYP3A4 and CYP3A5, meaning that for the same amount of holoenzyme, substantially greater amounts of CYP3A7 and CYP3A43 apoprotein were included in the comparison shown in Supplementary Figure 1, which may have inflated the apparent cross-reactivity of each antibody towards CYP3A7. Secondly, the amount of recombinant enzyme used for the immunoblots in Supplementary Figure 1 (0.5 pmol) exceeds many times the amounts seen in the native tissues, therefore the chances of nonspecific cross-reactivity are increased. Moreover, different
expression patterns were seen with each antibody suggesting preferential detection of two different antigens (Figures 1 and 6) and N-terminal sequencing of proteins affinity purified from cortical microsomes using the anti-CYP3A4 antibody showed that the predominant protein detected with this antibody in the cortex is CYP3A4 (*vide infra*).

**CYP3A forms are differentially expressed in the human brain**

Immunoblots using the affinity-purified polyclonal antibodies selectively targeted to CYP3A4 or CYP3A5 confirmed the presence of both CYP3A4 and CYP3A5 in all five brain regions analyzed, namely cortex, hippocampus, amygdala, basal ganglia and cerebellum (Figure 1). However, differential expression was observed between the cytosolic, mitochondrial and microsomal fractions between CYP3A4 and CYP3A5. While samples from only three individuals were available for detailed analysis, CYP3A expression patterns were consistent between samples with CYP3A signal detected in the same regional and sub-cellular locations. Expression levels varied slightly, with the sample with the longest post-mortem delay having slightly lower levels of CYP3A. No signal was detected for any P450 protein in immunoblots with pre-immune sera (data not shown).

CYP3A4 was detected in the microsomes, mitochondrial fraction and cytosol of all brain regions studied (Figure 1A). CYP3A5 was detected in the microsomal fractions of all five regions (Figure 1B). However, CYP3A5 was detected in the cytosolic fraction of only the cortex and hippocampus at significant levels and was absent from the mitochondrial fractions of any of the brain regions studied (Figure 1B).
Immunoblots with the affinity-purified antibody raised against CYP3A4 revealed the presence of an additional band in the microsomal fractions of all five brain regions studied which migrated more slowly than CYP3A4 in liver microsomes and with an estimated molecular weight approximately 10 kDa higher than CYP3A4 (Figure 1A). This band was not always observed, but did occur in the majority of CYP3A4 immunoblot experiments conducted on human brain microsomal samples. A “spiking” experiment was conducted to determine whether the second band was another CYP3A isoform or whether the signal was due to differential migration of CYP3A4 protein as seen previously for CYP2D6 in brain microsomal samples (Mann et al., 2008). Addition of comparable amounts of liver microsomes to brain microsomal samples resulted in the detection of only a single band for CYP3A4 protein, indicating that the second band observed in the brain microsomal fractions of CYP3A4 immunoblots was due to altered protein migration (Figure 2).

A lower molecular weight band was also observed in brain cytosol and microsomes probed with the antibody raised against CYP3A4 (Figure 1A). N-terminal sequencing of the antigen affinity-purified from cortical microsomes revealed that the first eight amino acids of the putative degradation product were WGFYDGQQ, which corresponds with the sequence located between amino acids 72-79 of the human CYP3A4 protein. N-terminal sequencing of the principal CYP3A4 band revealed the starting sequence of human CYP3A4 (ALIPDLAM). Notably, the corresponding sequences of CYP3A5 and CYP3A7 are: WGTYEGQL and WGIYDCQQ respectively (amino acids 72-79) and DLIPNLAV for both enzymes (amino acids 2-9).
CYP3A4 and CYP3A5 are predominantly expressed in the somatic region of neuronal cells

Immunohistochemical analysis using preimmune sera did not show any non-specific neuronal staining (Figure 3). CYP3A4 and CYP3A5 were expressed predominantly in the somatic region of neuronal cells, with expression frequently extending to the axonal hillock. Expression was also observed in neuronal axons (further down from the hillock and dendrites). The pattern of expression of the two forms differed within the same brain regions: CYP3A4 protein was detected from layer 3 through to layer 6 of the frontal cortex while CYP3A5 expression was observed from layer 2 through to layer 5 (Figure 3). CYP3A4 and 3A5 were both located in the hippocampus, where their expression appeared to be highest in the CA4 region (Figure 4). Both P450s were expressed in the amygdala and basal ganglia (Figure 5). By contrast, only CYP3A4 was detected in the cerebellum (Figure 5) at significant levels, where immunoreactivity was localized in large pyramidal cells as well as in the small neuronal cells of the cerebellar cortex.

CYP3A4 expression is elevated in microsomal fractions of frontal cortex from alcoholics

CYP3A expression in brain samples from controls and alcoholics was normalized to the α-tubulin signal, which was linear up to 30 μg (Supplementary Figure 2). CYP3A4 expression was higher in the microsomal fractions of the frontal cortex of brains from individuals with a history of alcohol abuse than in age- and sex-matched controls (Figure 6A) (P <0.05, 95 % CI), but no difference was seen in CYP3A4 protein expression between brain samples from alcoholics and controls in the mitochondrial fraction (data not shown). No difference was observed in the expression of CYP3A5 in either the microsomal or mitochondrial fractions of the human frontal
cortex between brains from alcoholics and controls (representative data for microsomal fractions is presented in Figure 6B).
Discussion

Despite their prominent role in drug metabolism, the study of CYP3A enzymes in the human brain has been hindered by the limited availability of brain tissue and of antibodies that can discriminate between CYP3A forms. Preliminary studies with form-specific short peptide antigens failed to generate antibodies of sufficient affinity (data not shown). Therefore in the current work, polyclonal antibodies raised to recombinant CYP3As were subject to affinity purification to obtain antibodies that showed markedly enhanced selectivity for CYP3A4 and CYP3A5. Immunoblots revealed expression of CYP3A4- and CYP3A5-related immunoreactivity to different extents in all five regions studied. The different patterns of expression seen with each antibody suggest preferential detection of two different antigens and the identity of the CYP3A4-immunoreactive band in the cortex was verified by N-terminal sequencing, supporting the contention that the antibodies are detecting the antigens against which they were raised. Limiting amounts of brain tissue prevented the use of this approach to confirm the identity of the anti-CYP3A4-reactive protein or anti-CYP3A5-reactive protein in other brain regions. However the fact that only CYP3A4, CYP3A5 and CYP3A43 have been detected in brain at the mRNA level to date (Murray et al., 1995; McFadyen et al., 1998; Agarwal et al., 2008) also supports the contention that the antibodies used here are detecting CYP3A4 and CYP3A5. CYP3A7 expression in the liver has been mainly observed in fetal and perinatal samples, whereas it seems to decrease significantly in adults (Lacroix et al., 1997; Tateishi et al., 1997; Nishimura et al., 2003; Stevens et al., 2003). To our knowledge, this study provides the first evidence for the expression of CYP3A5 at the protein level in human brain, consistent with previous results at the transcriptional level (McFadyen et al., 1998).
CYP3A protein expression in subcellular fractions varied, with CYP3A4 immunoreactivity detected in both the mitochondrial and microsomal fractions. By contrast CYP3A5 was absent from mitochondrial fractions but present in microsomes of all five regions tested, and in cytosolic fractions of the cortex and hippocampus. CYP3A expression has previously been observed in the mitochondrial fraction of whole rat brain extracts (Jayyosi et al., 1992). However, to our knowledge human brain CYP3A expression has only previously been assessed and reported in microsomes (Pai et al., 2002). Although cross-contamination may have occurred between subcellular fractions during sample processing, distinct patterns of expression were seen in the two fractions arguing against the hypothesis that apparent P450 expression in mitochondrial and soluble fractions was due to contamination with microsomes. Importantly, it is now recognized that many “microsomal” P450s are also expressed in mitochondria, cytosol and plasma membrane (Neve and Ingelman-Sundberg, 2008; Avadhani et al., 2011). Import into the mitochondrion involves an initial transit through the cytosol, which may explain the cytosolic localization of CYP3A4 in the present study, given the clear signal observed in the mitochondrial fraction. Moreover, brain P450 localization in soluble fractions might be ascribed to limited heme availability in this organ, which may impair enzyme insertion into the ER membrane (Meyer et al., 2002).

Three bands were clearly evident in frontal cortex microsomes incubated with anti-CYP3A4 antibody. The middle band was identified as full-length CYP3A4 by N-terminal sequencing while the lower one appears to be a proteolysis product missing the first 71 residues (not prominent in the pooled sample used for Figure 2) and may correspond to the functional truncated form found previously (Jeon et al., 2008). Variable migration of brain P450 proteins compared to the same forms in liver has been observed previously (Mann et al. 2008).
hypothesize that the upper band may represent protein subject to post-translational modification. Addition of comparable amounts of human liver microsomal protein to frontal cortex microsomes led to the loss of the upper band. We speculate that addition of liver microsomes led to restoration of the normal migration pattern due to the presence of an enzyme in liver microsomes which removed the post-translational modification. Further experiments are required to determine the basis to the slower migration of the upper band.

CYP3A isoforms were distributed differently between and within brain regions with anti-CYP3A4 immunoreactivity detected from layer 3 through to layer 6 of the frontal cortex, and anti-CYP3A5 immunoreactivity observed from layer 2 through to layer 5. Consistent with these observations, CYP3A4, CYP3A5 and CYP3A43 (but not CYP3A7) have been detected at the mRNA level in human cortex (Murray et al., 1995; McFadyen et al., 1998; Agarwal et al., 2008). Recently CYP3A4 has also been detected at the mRNA and protein level in neurons and endothelial cells in temporal lobe from patients receiving anticonvulsants that are known inducers of hepatic CYP3A4 (Ghosh et al., 2010; Ghosh et al., 2011); however no clear evidence for expression in microvessels was seen here or in a previous study (Dauchy et al., 2008).

The localization of both CYP3A4 and CYP3A5 in the pyramidal cell layer of hippocampal regions CA1-CA4 (Figure 4) accords with previous results at the transcriptional level. CYP3A-related mRNA was found in hippocampal regions CA1-CA3 (Pai et al., 2002), CYP3A43 mRNA was detected in the pyramidal neurons of the hippocampus (Agarwal et al., 2008), and CYP3A5 was detected but not quantified in hippocampus by real time-PCR (Dutheil et al., 2009). CYP3A4 and CYP3A5 were also both expressed in pyramidal neurons of the basal ganglia (Figure 5) in accordance with expression of both forms at the transcriptional level in basal
ganglia (McFadyen et al., 1998). Both CYP3A4 and CYP3A5-related immunoreactivity was seen in the amygdala by both immunoblotting and immunohistochemistry. To our knowledge, this is the first report of expression of any CYP3A form in human amygdala at either the protein or mRNA level. However, cyp3a11 protein has been detected in murine amygdala (Meyer et al., 2009).

CYP3A4 was detected immunohistochemically in the cerebellum at significant levels in the pyramidal and granular cell layers and in small neuronal cells of the cerebellar cortex. Agarwal et al. (2008) reported CYP3A4 but not CYP3A43 mRNA in pyramidal cells of the cerebellum, with CYP3A43 localized instead to granular cells and occasional cells in the molecular layer. While CYP3A5 has not yet been detected at the mRNA level in cerebellum, trace signal was detected here with the antibody raised to CYP3A5; however the long thin structures with which it was associated could not be positively identified. This result is consistent with the weak band that was observed for CYP3A5 in cerebellum by immunoblotting, suggesting the greater sensitivity of the Odyssey imaging system allows detection of the minimal expression of CYP3A5 in this tissue.

The shortage of human tissue and lengthy postmortem delays encountered with available brain samples obviated any investigation of whether the CYP3A forms detected here were functionally active. Regio-specific expression and differential induction of CYP3A in the brain may have implications for the site-specific disposition of xenobiotics and therefore influence the efficacy and/or toxicity of drugs (Britto and Wedlund, 1992; Pai et al., 2002). CYP3A4 but not CYP3A5 expression appeared to be upregulated in the frontal cortex of human alcoholic brain. Previous studies suggested that CYP3A expression is induced by ethanol in liver in vivo and ex vivo in primary hepatocytes (Hoshino and Kawasaki, 1995; Kostrubsky et al., 1995) and in the intestine.
of moderate alcohol consumers (Liangpunsakul et al., 2005). However, to our knowledge, this is the first report of induction of CYP3A by alcohol in the human brain. It was beyond the scope of this study to confirm the mechanism underlying the observed induction of CYP3A4. However, one of the known mechanisms of ethanol-mediated P450 induction is enzyme stabilization, which has been observed both in liver and extrahepatically (Song et al., 1988; Koop and Tierney, 1990). Notably, this type of ethanol-mediated induction has also been observed with CYP3A4 expressed in HepG2 cells (Feierman et al., 2003). CYP3A metabolism of both endogenous and exogenous compounds was inhibited by alcohol in human liver (Patki et al., 2004). Induction of CYP3A4 in the frontal cortex may represent a homeostatic response to alcohol inhibition of CYP3A activity, alterations in CYP3A activity has been proposed to affect steroid-mediated signaling in the brain (Meyer and Gehlhaus, 2010). Changes in the dopaminergic system have also been linked to altered hepatic CYP3A expression in animals (Wojcikowski et al., 2007). Since chronic alcohol consumption can affect the dopaminergic system, it is conceivable that human CYP3A4 expression may be altered by the long-term effects of alcohol on neuronal signaling. Induction of CYP activity could affect localized drug or neurotransmitter metabolism with consequences for neural cell function. Further investigation is required to establish the mechanism underlying the changes in CYP3A4 expression in human brain and the possible biological significance of this effect.

In summary, this study has shown that CYP3A4 and CYP3A5 are expressed at the protein level in a number of human brain regions. The differential compartmentalization and response to chronic alcohol exposure seen here suggests that, assuming the proteins detected in the brain are active, CYP3A4 and CYP3A5 may have functionally different roles in the metabolism of endo- and xenobiotics.
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Authorship contributions

Participated in research design: Booth-Depaz, Wilce, Gillam

Conducted experiments: Booth-Depaz, Toselli

Contributed new reagents or analytic tools: Wilce

Performed data analysis: Booth Depaz, Toselli, Gillam

Wrote or contributed to the writing of the manuscript: Booth-Depaz, Toselli, Gillam, Wilce
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Figure legends

Figure 1

**CYP3A protein expression in human brain fractions**

Polyclonal, affinity-purified antibodies raised against recombinant CYP3A4 (A) and CYP3A5 (B) were used to assess CYP3A4 (A) and CYP3A5 (B) expression in cortex, hippocampus, basal ganglia, amygdala and cerebellum of human control samples. A. CYP3A4 expression was observed in the microsomal, mitochondrial and cytosolic fractions of all brain regions, but to a significantly lesser extent in the cerebellar mitochondrial fraction and cytosolic fractions of amygdala and hippocampus. B. CYP3A5 was observed in the microsomal fractions of all five brain regions, as well as the cytosolic fraction of the cortex and hippocampus. No CYP3A5 expression was observed in the mitochondrial fractions of any of these regions. Representative blots are shown with subcellular fractions from one individual (S3, Table 1), chosen to illustrate both the slower migrating CYP3A4 band and the lower molecular weight CYP3A4 proteolysis product. Twenty micrograms of total protein were loaded in each lane for brain samples, while one microgram of recombinant bacterial membranes was loaded for each control, containing either 0.4 pmol CYP3A4 holoenzyme or 0.18 pmol CYP3A5 holoenzyme. Abbreviations are: CTX, cortex; HP, hippocampus; BG, basal ganglia; AM, amygdala; CB, cerebellum; HL, human liver microsomes; 3A4, recombinant CYP3A4; 3A5, recombinant CYP3A5.

Figure 2

**Resolution of irregular migration of CYP3A4 in microsomal brain samples by addition of liver microsomes**
Abbreviations are: Rec 3A4, recombinant CYP3A4 protein; HL µs, liver microsomes; Ctx µs, cortex microsomes; HL+Ctx µs, human cortex microsomes to which liver microsomes had been added; Ctx Mito, cortex mitochondrial sample. CYP3A4 immunoblotting revealed the presence of a more slowly migrating band in the microsomal fractions of cortex but not liver. Uniform migration of both brain and liver CYP3A4 was observed after addition of human liver microsomal protein to human microsomal cortex samples pooled from three individuals (S1-3, table 1), suggesting that the more slowly migrating band observed in human brain samples was CYP3A4 and not an alternative translation product. Variable migration of brain P450 proteins has been observed previously (Mann et al. 2008). An equal amount (20 μg) of total protein was loaded in each lane. The migration of molecular weight markers is indicated at the side of the blot. The lower molecular weight CYP3A4 proteolysis product is barely detectable in the cortex microsomal sample above the 40 KDa marker due to its limited abundance in two of the three brain samples. Slight differences in the relative mobility of CYP3A4 bands between the blots shown in Figure 1A and 2 can be ascribed to minor alterations in sample composition and gel preparation between the two experiments.

Figure 3

Immunohistochemical detection of CYP3A4 and CYP3A5 expression in the human frontal cortex

CYP3A4 protein was detected in neurons of layer 3 through to layer 6 of the frontal cortex. In contrast, CYP3A5 expression was observed in layers 2 through to layer 5. The expression of CYP3A4 and CYP3A5 occurred predominantly in the somatic region of neuronal cells.
Expression was also observed in neuronal axons and dendrites. A and B: negative controls (preimmune sera) for CYP3A4 and CYP3A5 respectively; C, E, G and I: layers 3-6 treated with anti-CYP3A4 antibody; D, F, H and J: layers 2-5 treated with anti-CYP3A5 antibody. Scale bar = 50 μm.

**Figure 4**

**Immunohistochemical detection of CYP3A4 and CYP3A5 expression in the human hippocampus**

CYP3A4 and 3A5 were both located throughout the hippocampus, however expression appeared to be highest in the CA4 region in both cases. A, C, E and G: CA1-4 treated with anti-CYP3A4 antibody; B, C, F and H: CA1-4 treated with anti-CYP3A5 antibody. A’-H’ represent the same regions treated with the respective pre-immune serum as a negative control. Scale bar = 50 μm.

**Figure 5**

**Immunohistochemical detection of CYP3A4 and CYP3A5 expression in the human amygdala, basal ganglia and cerebellum**

A, C, and E: Brain regions treated with anti-CYP3A4 antibody; B, C, F: Brain regions treated with anti-CYP3A5 antibody. A’-F’ show the same regions treated with the respective pre-immune serum as a negative control. Scale bar = 50 μm. Both P450s were expressed in the amygdala (A,B) and basal ganglia (C, D). In contrast, only CYP3A4 was detected at significant levels in the cerebellum (E), where it was localized in the large pyramidal cells of the cerebellum.
as well as in the granular layer and the small neuronal cells of the cerebellar cortex. CYP3A5 was detected at only trace levels in human cerebellum (F) in the immunohistochemical analysis. The elongated structures showing CYP3A5 localization could not be positively identified.

**Figure 6**

**CYP3A4 expression is elevated in microsomal fractions of the alcoholic human frontal cortex**

CYP3A4 and CYP3A5 expression was assessed in microsomal fractions of the frontal cortex of six individual control and six individual alcoholic case-matched human brain samples using affinity-purified antibodies raised against recombinant CYP3A4 (A) and CYP3A5 (B). An equal amount (20 μg) of total protein was loaded in each lane. C: Individual sample intensities were integrated using the LiCor software, corrected against α-tubulin and utilized for statistical analysis. Data were subjected to paired Student-Newman-Keuls t-test analysis with a confidence interval (CI) of 95%. Data are expressed as mean ± SEM. CYP3A4 expression in samples from alcoholics was significantly elevated over expression in controls (*, P < 0.05, 95% CI, n = 6). Differential expression of CYP3A4 protein between alcoholic and control brains was not observed in the mitochondrial fraction (data not shown). No significant differences were seen between samples from alcoholics and controls with respect to CYP3A5 expression.
Table 1. Patient history details for control brain samples used for studies of the regio-selective expression of CYP3A proteins.

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**Table 2. Clinical details and characteristics pertaining to alcoholic and control samples.**

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Differential expression of human cytochrome P450 enzymes from the CYP3A subfamily in the brains of alcoholics and drug free controls

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Drug Metabolism and Disposition

Supplementary information
Supplementary Figure 1. Cross-reactivity of affinity-purified anti-CYP3A4 (A) and anti-CYP3A5 (B) antibodies towards recombinant CYP3A4, CYP3A5, CYP3A7 and CYP3A43 expressed in bacterial membrane fractions.

Bacterial membranes containing recombinant CYP3A forms were subjected to SDS-PAGE and immunoblotting with antibodies raised against purified recombinant CYP3A4 (A) and 3A5 (B). A. Lane 1, purified CYP3A4; lanes 2-5, bacterial membranes containing recombinant CYP3A4, CYP3A5, CYP3A7 and CYP3A43; lane 6, bacterial membranes containing recombinant human NADPH-P450 reductase; lane 7, bacterial membranes from cells transformed with the pCW vector alone. B. Lanes 1-4, bacterial membranes containing recombinant CYP3A4, CYP3A5, CYP3A7 and CYP3A43; lane 5, bacterial membranes containing recombinant human NADPH-P450 reductase; lane 6, membranes from bacteria.
transformed with the pCW vector alone. The amount of sample loaded was 0.5 pmol of P450 enzyme, 1.9 pmoles of recombinant reductase and 4.8 µg of total protein for pCW. The cross-reactivity of the anti-CYP3A4 antibody was expressed as the ratio of the band intensity for each form over that for CYP3A4 and was 0.51 and 0.93 for CYP3A5 and 3A7 respectively. The cross-reactivity of the antibody raised against recombinant CYP3A5 for each other form was expressed as the ratio of the blot intensity for each form over that for CYP3A5 and was 0.51 and 0.81 for CYP3A4 and 3A7 respectively.
Supplementary Figure 2. Linearity of α-tubulin signal in microsomes from human frontal cortex. (A) Detection of α-tubulin signal in increasing amounts of frontal cortex microsomal protein in the range of 0 to 30 µg. Integrated intensity was calculated using the LiCor Odyssey software and expressed in arbitrary units of kilo counts. The line of best fit was obtained by linear regression of the integrated intensity values and shows an $R^2$ value of 0.8747. (B) Immunoblot showing the linearity of α-tubulin detection under the conditions used.