The Effects of Ethanol and Nicotine on Hepatic and Brain CYP2 Family Enzymes in African Green Monkeys

by

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Pharmacology and Toxicology
University of Toronto

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Abstract
Cytochromes P450 (CYPs) metabolize a vast array of xenobiotics, including many drugs and toxins. Induction or downregulation of the CYPs can have important consequences such as changes in drug efficacy and altered susceptibility to toxicity. Our study investigated the independent and combined effects of ethanol and nicotine on hepatic and/or brain levels of CYP2E1, CYP2B6 and CYP2A6 in African green monkeys. Monkeys were randomized into four groups (N = 10/group): an ethanol-only group, a nicotine-only group, an ethanol + nicotine group and a control (no drug) group. Ethanol (10% ethanol in sucrose solution) was voluntarily self-administered by the monkeys and nicotine was given as subcutaneous injections (0.5 mg/kg bid). Protein levels and/or in vitro activity were assessed in liver and brain tissue. Also, in vivo pharmacokinetics for chlorzoxazone (metabolized selectively by CYP2E1) and nicotine (metabolized primarily by CYP2A6 and to a lesser extent CYP2B6) were assessed. Hepatic CYP2E1 protein levels, in vitro hepatic CYP2E1 activity and in vivo chlorzoxazone metabolism were increased by ethanol and nicotine, alone and in combination. Hepatic CYP2B6 protein levels and in vitro hepatic CYP2B6 activity were increased by ethanol alone or combined ethanol and nicotine exposure, but were unaffected by nicotine alone. Hepatic CYP2A6 protein levels and in vitro hepatic CYP2A6 activity were decreased by nicotine alone or combined ethanol and nicotine exposure, but unaffected by ethanol alone. Chronic nicotine resulted in higher nicotine plasma levels achieved after nicotine administration, consistent with decreased CYP2A6 activity. Ethanol, alone or combined with nicotine, resulted in lower nicotine plasma levels, an effect that was not mediated by changes in CYP activity. Protein levels of CYP2E1 and CYP2B6 were
induced in specific regions and cells in the brain as a result of ethanol self-administration, nicotine treatment and the combined exposure to both drugs. In summary, ethanol and nicotine can alter the expression and/or activity of several important CYP2 family enzymes in primate liver and/or brain.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>3HC</td>
<td><em>Trans</em>-3’-hydroxycotinine</td>
</tr>
<tr>
<td>6-OH-CZN</td>
<td>6-hydroxycarboxazone</td>
</tr>
<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>AGM</td>
<td>African green monkey</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>CEBP</td>
<td>CCAAT-box/enhancer binding protein</td>
</tr>
<tr>
<td>CSE</td>
<td>Chronic solvent induced encephalopathy</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>CZN</td>
<td>Chloroxazone</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DR4</td>
<td>Direct repeat separated by four nucleotides</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor-α</td>
</tr>
<tr>
<td>FMO3</td>
<td>Flavin-containing monooxygenase 3</td>
</tr>
<tr>
<td>GSTM1</td>
<td>Glutathione s-transferase M1</td>
</tr>
<tr>
<td>HLM</td>
<td>Human liver microsomes</td>
</tr>
<tr>
<td>HNF1-α</td>
<td>Hepatocyte nuclear factor-1α</td>
</tr>
<tr>
<td>HNF4-α</td>
<td>Hepatocyte nuclear factor-4α</td>
</tr>
<tr>
<td>hnRNPA1</td>
<td>Heterogeneous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>LRH-1</td>
<td>Liver receptor homolog-1</td>
</tr>
<tr>
<td>MOCA</td>
<td>4,4-methylenebis (2-chloroaniline)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCO</td>
<td>Nicotine c-oxidation</td>
</tr>
<tr>
<td>NDEA</td>
<td>N-Nitrosodiethylamine</td>
</tr>
<tr>
<td>NFATc</td>
<td>Fos-related antigenic transcription factor</td>
</tr>
<tr>
<td>NNK</td>
<td>4-(methylN-nitrosamino)- 1-(3-pyridyl)-1-butanone</td>
</tr>
<tr>
<td>NOR1</td>
<td>Neuron-derived orphan receptor 1</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor (erythroid-derived 2)-like 2</td>
</tr>
<tr>
<td>Nur77</td>
<td>Neuron-derived clone 77</td>
</tr>
<tr>
<td>Nurr1</td>
<td>Nuclear receptor related</td>
</tr>
<tr>
<td>Oct-1</td>
<td>Octamer transcription factor-1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor-γ coactivator 1α</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PREM</td>
<td>Phenobarbital-responsive enhancer module</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>THioTEPA</td>
<td>N,N’N’-triethylenethiophosphoramide</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>Vd</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>XREM</td>
<td>Xenobiotic responsive enhancer module</td>
</tr>
</tbody>
</table>
Section 1: Introduction

Statement of Research Problem

Cytochrome P450 (CYP) enzymes are responsible for metabolizing a large variety of xenobiotics, including many drugs and toxins. Certain CYP isoforms are highly inducible and their activity is greatly influenced by environmental factors, such as diet and exposure to various chemicals (Zanger and Schwab, 2013). The induction or downregulation of CYPs can significantly alter xenobiotic metabolism and contribute to observed interindividual differences in drug response and susceptibility to toxins (Pelkonen et al., 2008). Interindividual variability in response to xenobiotics poses a substantial clinical problem and can often hinder successful drug development, drug therapy and toxicity risk assessments. A better understanding of the environmental factors that influence CYP expression and activity will improve our ability to predict which individuals will benefit from certain drug treatments and which individuals will be more susceptible to toxicity from specific compounds.

Elevated levels of CYP2E1 and CYP2B6 have been detected in the livers and/or brains of heavy alcohol users and smokers, whereas decreased activity of hepatic CYP2A6 has been detected in smokers (Tsutsumi et al., 1989; Benozwitz and Jacob, 2000; Hesse et al., 2000; Benowitz et al., 2003; Howard et al., 2003; Miksys et al., 2003). We hypothesize that the altered levels of these CYPs in alcohol users and smokers are due to ethanol and nicotine exposure, respectively. A large proportion of the population is exposed to ethanol and nicotine, often in combination. Given the potential adverse consequences of altered CYP-metabolism, it is vital that we determine the independent and combined effects of ethanol and nicotine on CYP2E1, CYP2B6 and CYP2A6 levels in liver and/or brain.
A causal relationship between ethanol/nicotine exposure and altered expression of CYPs in liver and brain is difficult to demonstrate in humans. However, the effects of ethanol and nicotine on CYP levels can be studied in non-human primates, both in vivo and ex vivo, and can be used to predict the effects in humans. Determining the impact of ethanol and nicotine on CYP2E1, CYP2B6 and CYP2A6 levels in monkey liver and/or brain will provide insight into the magnitude and potential consequences of CYP induction/downregulation by ethanol and nicotine in humans.

Review of the literature

1. Cytochromes P450 (CYPs)

1.1 Oxidative metabolism by CYPs

CYPs are a superfamily of enzymes that metabolize a wide variety of xenobiotics including drugs, industrial chemicals and environmental toxins, as well as endogenous compounds, such as steroids, fatty acids and vitamins (Coon, 2005). CYPs are heme-containing monooxygenases that catalyze the oxidation of organic compounds, usually making them more hydrophilic to facilitate further biotransformation and excretion. The monooxygenation reaction mediated by most CYPs generally involves the addition of oxygen to an organic substrate to produce water and a monooxygenated metabolite (Coon, 2005). This reaction requires the transfer of two electrons from nicotinamide adenine dinucleotide phosphate (NADPH) mediated by the redox partner NADPH-cytochrome P450 oxidoreductase (Lewis and Hlavica, 2000). Depending on the CYP isoform, the second of the two electrons required could be donated by nicotinamide adenine dinucleotide (NADH) via cytochrome b5 (Lewis and Hlavica, 2000). Most mammalian CYPs
and their redox partners are membrane-bound, localized to the outer face of the endoplasmic reticulum and the inner membrane of the mitochondria (Stier, 1976).

1.2 Nomenclature for CYPs

A nomenclature system has been developed to group CYPs into families and subfamilies based on amino acid sequence similarities (Nelson, 2005; Dauchy et al., 2008). A CYP family consists of enzymes with greater than 40% amino acid sequence homology and is designated by a number (e.g. CYP2). A subfamily consists of enzymes with greater than 55% amino acid homology and is designated by a letter (e.g. CYP2A and CYP2B are subfamilies of the CYP2 family). Individual CYP enzymes within a subfamily are designated by a subsequent number (e.g. CYP2A6 and CYP2A13 are specific enzymes belonging to the CYP2A subfamily).

To date, 57 functional CYP genes belonging to 18 families and 42 subfamilies have been identified in humans (Nelson, 2009). The CYP1, CYP2 and CYP3 families are mainly responsible for the metabolism of drugs and other xenobiotics, but they also metabolize many endogenous compounds (Zanger and Schwab, 2013). CYPs from the other families are primarily involved in endogenous processes, such steroid biosynthesis and fatty acid metabolism (Preissner et al., 2010).

1.3 Hepatic CYPs

CYPs are found in virtually all tissues, with the highest abundance and largest number of CYP isoforms present in the liver. As seen in Figure 1, CYP content in the liver is mostly comprised of xenobiotic-metabolizing enzymes from CYP families 1-3 (Emoto et al., 2006).
Figure 1. Relative expression of major CYP isoforms in human liver. Expression of CYP isoforms in human liver as a percentage of total CYP content (Rendic and Di Carlo, 1997; Emoto et al., 2006).

CYPs are also present in the intestines, lung, kidney, brain, adrenal gland, gonads, heart, nasal and tracheal mucosa, bone marrow and skin; however, the content of xenobiotic-metabolizing CYPs is generally much lower in these extra-hepatic tissues compared to the liver (Ding and Kaminsky, 2003; Nishimura et al., 2003; Yengi et al., 2003; Pavek and Dvorak, 2008; Chaudhary et al., 2009). While extra-hepatic metabolism may have important effects locally within a tissue, systemic metabolic clearance of most xenobiotics occurs predominantly in the liver (Ding and Kaminsky, 2003).

The expression of most xenobiotic-metabolizing CYPs in the liver can be induced or downregulated in response to a variety of chemicals and/or certain pathophysiological states (Pelkonen et al., 2008). Changes in the expression of hepatic CYPs can alter the metabolism of drugs, toxins and other chemicals, which can have important clinical consequences such as changes in drug efficacy and altered susceptibility to toxicity. The induction or downregulation of hepatic CYPs may also impact the metabolism of endogenous substrates, affecting the circulating levels of steroids or signaling molecules.
1.4 Brain CYPs

To date, 41 of the 57 human CYP transcripts have been identified in the brain (Dauchy et al., 2008; Dutheil et al., 2009). However, protein expression and/or activity of only a few xenobiotic-metabolizing CYPs (e.g. CYP1A, CYP1B, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A subfamilies) have been studied to any great extent in the brain (Baker et al., 2007). The total CYP levels in the brain are low, approximately 0.5–2% of those in the liver, therefore it is unlikely that brain CYPs substantially influence systemic drug and metabolite levels (Hedlund et al., 2001). However, CYPs are heterogeneously expressed in the brain, concentrated in specific regions and cell types where they can potentially have a considerable impact on metabolism in certain brain microenvironments or the brain as a whole (Ferguson and Tyndale, 2011).

The activity of brain CYPs has been demonstrated \textit{in vitro} and \textit{in vivo}, showing similar substrate profiles and substrate affinities compared to their respective hepatic CYP isoforms (Hedlund et al., 2001; Ferguson and Tyndale, 2011). Brain CYPs are inducible by many of the same compounds that induce hepatic CYPs, such as the induction of CYP2B enzymes by phenobarbital (Schilter et al., 2000; Lee et al., 2006a), and induction of CYP2E1 by acetone (Sánchez-Catalán et al., 2008). However, induction can also be organ-specific with differential induction of CYPs in the liver and brain. For example, CYP2D enzymes are essentially uninducible in the liver, but can be induced by compounds such as nicotine and the neuroleptic drug clozapine in the brain (Hedlund et al., 1996; Mann et al., 2008). The regulation of brain CYPs is complex, with region- and cell-specific induction. For instance, in monkeys, chronic nicotine treatment induced CYP2D enzyme levels in the putamen, substantia nigra, brainstem, cortex, hippocampus and cerebellum, whereas other brain regions, such as the nucleus
accumbens and globus pallidus, were unaffected (Mann et al., 2008). Cell specificity of CYP2D induction by nicotine was apparent in the cerebellum, where CYP2D was increased in Purkinje cells, but not in cells in the molecular or granular layer (Mann et al., 2008).

The induction or downregulation of brain CYPs can potentially affect the local metabolism of xenobiotics in certain brain microenvironments or the brain as a whole. Changes in CYP activity in the brain can alter sensitivity to centrally acting drugs and toxins, affecting drug efficacy and susceptibility to neurotoxicity (Khokhar and Tyndale, 2011; 2012). CYPs also contribute to the biotransformation of important brain signaling molecules, such as neurotransmitters and neurosteroids (Hiroi et al., 1998; Badawi et al., 2001; Fradette et al., 2004; Kishimoto et al., 2004; Sridar et al., 2011). Alterations in brain CYP levels could potentially modulate brain function and lead to cognitive and/or behaviour changes (Fang et al., 2012).

2. Cytochrome P450 2E1 (CYP2E1)

2.1 Expression of CYP2E1 in humans and other species

CYP2E1 is the only enzyme belonging to the CYP2E subfamily present in humans, monkeys, rats and mice (http://drnelson.uthsc.edu/CytochromeP450.html). Human CYP2E1 is primarily expressed in the liver, but it is also found in extra-hepatic tissues such as the brain, lung, nasal mucosa and gastrointestinal tract (Ding and Kaminsky, 2003; Dutheil et al., 2009; Leclerc et al., 2010). In the human brain, CYP2E1 protein has been detected in multiple regions, with varying levels of expression among regions and cell types (Howard et al., 2003). The CYP2E1 gene is highly conserved across species. The deduced amino acid sequence for human CYP2E1 shares 94%, 79% and 78% similarity with monkey, rat and mouse CYP2E1, respectively (% sequence identity determined using Clustal W, http://www.genome.jp/tools/clustalw)
2.2 CYP2E1 substrates and inhibitors

CYP2E1 metabolizes a diverse array of exogenous and endogenous substrates, most of which are small, low molecular weight molecules (Lieber, 1997; Porubsky et al., 2008) (Table 1). A large proportion of CYP2E1 substrates are industrial solvents and chemicals that are metabolically activated by CYP2E1 to toxic or carcinogenic metabolites (Bolt et al., 2003; Trafalis et al., 2012); consequently, CYP2E1 is of great interest in the field of occupational and environmental medicine.

Table 1. Examples of CYP2E1 substrates

<table>
<thead>
<tr>
<th>Drugs</th>
<th>EXOGENOUS</th>
<th>Toxins</th>
<th>ENDOGENOUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analgesic</td>
<td>Acetaminophen</td>
<td>Industrial chemicals</td>
<td>Nitrosamines</td>
</tr>
<tr>
<td>Anesthetic gases</td>
<td>Halothane</td>
<td>Acrylonitrile</td>
<td>Nitrodimethylamine</td>
</tr>
<tr>
<td></td>
<td>Enflurane</td>
<td>Acrylamide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sevoflurane</td>
<td>Aniline</td>
<td></td>
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<tr>
<td>Cessation aid</td>
<td>Butadiene</td>
<td>Methyl chloride</td>
<td>Organic solvents</td>
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<tr>
<td></td>
<td></td>
<td>Phenol</td>
<td>Benzene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vinyl chloride</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vinyl bromide</td>
<td>Chloroform</td>
</tr>
<tr>
<td>Drugs of abuse</td>
<td>Ethanol</td>
<td>Industrial chemicals</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>Muscle relaxant</td>
<td>Chlorzoxazone</td>
<td>Acrylamide</td>
<td>Diethylether</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenol</td>
<td>Hexane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vinyl chloride</td>
<td>Pyridine</td>
</tr>
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<td></td>
<td></td>
<td>Vinyl bromide</td>
<td>Styrene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acrylamide</td>
<td>Trichloroethane</td>
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<td></td>
<td></td>
<td>Aniline</td>
<td>Toluene</td>
</tr>
</tbody>
</table>

Most xenobiotics cleared by the CYP system are metabolized by multiple CYP isoforms. When the metabolism of a substrate involves a pathway that is primarily mediated by a single CYP to generate a specific metabolite, this substrate can potentially be used as a probe drug to assess that CYP’s activity (Yuan et al., 2002). Chlorzoxazone, a skeletal muscle relaxant, is
metabolized to 6-hydroxychlorzoxazone via a pathway that is almost exclusively mediated by CYP2E1 (>90%) (Lucas et al., 1999). The rate of chlorzoxazone metabolism to 6-hydroxychlorzoxazone has been established as a phenotypic measure of CYP2E1 activity in vitro and in vivo (Lucas et al., 1999).

Examples of CYP2E1 inhibitors and their modes of action are listed in Table 2. Based on their mode of action, CYP inhibitors can be classified into four main types: competitive, non-competitive, uncompetitive and mechanism-based (Zhang and Wong, 2005).

### Table 2. Examples of CYP2E1 inhibitors

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>MODE OF INHIBITION</th>
<th>Ki in HLM(^a) (µM)</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Methylpyrazole</td>
<td>Competitive</td>
<td>2</td>
<td>(Collom et al., 2008)</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
<td>Mechanism based</td>
<td>10-30</td>
<td>(Yamazaki et al., 1992)</td>
</tr>
<tr>
<td>Diallyl sulphide</td>
<td>Mechanism based</td>
<td>118</td>
<td>(Brady et al., 1991a)</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>Mechanism based</td>
<td>Not known</td>
<td>(Brady et al., 1991b)</td>
</tr>
</tbody>
</table>

\(^a\) Ki derived from experiments in human liver microsomes (HLM)

Competitive inhibitors bind close to, or within, the substrate-binding domain on the enzyme and limit substrate access to the catalytic site. Non-competitive inhibitors bind to an allosteric site on the enzyme, causing altered substrate binding and metabolism. Uncompetitive inhibitors bind to the substrate-enzyme complex, but not the enzyme alone, hindering the metabolism of the substrate. Mechanism-based inhibitors are substrates for the target enzyme; the inhibitor is metabolized to a reactive intermediate that covalently binds the enzyme rendering it inactive.
2.2 Generation of reactive oxygen species by CYP2E1

As discussed, the typical CYP-mediated monooxygenation reaction involves the addition of oxygen to an organic substrate (RH) to produce water and a monooxygenated metabolite (ROH) (Reaction 1). Sometime this reaction can become “uncoupled” and oxygen is reduced to reactive oxygen species (ROS), such as superoxide anion radical (O$_2^•$) and hydrogen peroxide (H$_2$O$_2$), instead of being added to a substrate (Reaction 2 and 3). Relative to other CYP enzymes, CYP2E1 is more prone to these uncoupled reactions and exhibits a high rate of ROS production, even in the absence of substrate (Caro and Cederbaum, 2004).

\[
\begin{align*}
\text{ROH} + \text{O}_2 + \text{NADPH} + \text{H}^+ & \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+ & (1) \\
2\text{O}_2 + \text{NADPH} & \rightarrow 2\text{O}_2^• + \text{NADP}^+ + \text{H}^+ & (2) \\
\text{O}_2 + \text{NADPH} + \text{H}^+ & \rightarrow \text{HOOH} + \text{NADP}^+ & (3)
\end{align*}
\]

ROS can interact with cellular macromolecules to cause lipid peroxidation, protein denaturation, DNA damage and cell death (Caro and Cederbaum, 2004). The overexpression of CYP2E1 in liver cell lines is associated with increased ROS production, cellular injury and cytotoxicity. (Ekström and Ingelman-Sundberg, 1989; Dai et al., 1993; Wu and Cederbaum, 2001; Hodges et al., 2007). The essential role of CYP2E1 in the generation of ROS-mediated cell damage was demonstrated with specific CYP2E1 inhibitors that blocked ROS production and provided protection against cellular damage (Wu and Cederbaum, 2001; Hodges et al., 2007).
2.3 Regulation of CYP2E1

2.3.1 Hepatic CYP2E1

The regulation of hepatic CYP2E1 is complex, involving transcriptional, post-transcriptional and post-translational mechanisms. CYP2E1 can be induced by a variety of drugs, chemicals and pathophysiological states (Lieber, 1997) (Table 3).

### Table 3. Examples of hepatic CYP2E1 inducers

<table>
<thead>
<tr>
<th>INDUCER</th>
<th>MECHANISM OF INDUCTION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>Transcriptional, protein stabilization</td>
<td>(Ronis et al., 1993)</td>
</tr>
<tr>
<td>Acetone</td>
<td>Protein stabilization</td>
<td>(Song et al., 1989)</td>
</tr>
<tr>
<td>Benzene</td>
<td>Transcription and/or increased mRNA stability</td>
<td>(González-Jasso et al., 2003)</td>
</tr>
<tr>
<td>Toluene</td>
<td>Transcription and/or increased mRNA stability</td>
<td>(González-Jasso et al., 2003)</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Protein stabilization</td>
<td>(Chien et al., 1997)</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Protein stabilization</td>
<td>(Tamási et al., 2003)</td>
</tr>
<tr>
<td>Pyrazole</td>
<td>Protein stabilization</td>
<td>(Zerilli et al., 1998)</td>
</tr>
<tr>
<td>Imidazole</td>
<td>Increased translation</td>
<td>(Tamási et al., 2003)</td>
</tr>
<tr>
<td>Pyridine</td>
<td>Increased protein translation</td>
<td>(Wu et al., 1997)</td>
</tr>
<tr>
<td>Starvation</td>
<td>Increased mRNA stability</td>
<td>(Hu et al., 1995)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Increased mRNA stability</td>
<td>(Song et al., 1987)</td>
</tr>
<tr>
<td>Obesity</td>
<td>Increased mRNA stability</td>
<td>(Khemawoot et al., 2007)</td>
</tr>
</tbody>
</table>

Several transcription factors regulate CYP2E1 gene expression in the liver, including hepatocyte nuclear factor 1α (HNF1-α) (Cheung et al., 2003), nuclear factor of activated t-cells c (NFATc) (Wang et al., 2010a), STAT6 (Wang et al., 2010a), GATA-4 (Shadley et al., 2007), Liver receptor homolog-1 (LRH-1; NR5A2) (Shadley et al., 2007) and CCAAT-enhancer-
binding proteins (CEBPs) (Kellicen and Tindberg, 2004). However, the induction of hepatic CYP2E1 protein levels and activity by typical inducers does not usually involve transcriptional activation (Gonzalez, 2006).

Hepatic levels of CYP2E1 protein and mRNA are highly induced by pathophysiological states such as diabetes, prolonged starvation and obesity (Song et al., 1987; Hu et al., 1995; Khemawoot et al., 2007). The increase in CYP2E1 mRNA observed with these pathological states is not accompanied by an enhanced rate of CYP2E1 transcription, suggesting that CYP2E1 mRNA is being stabilized against degradation (Song et al., 1987). CYP2E1 mRNA stabilization may be a mechanism by which CYP2E1 protein levels are induced (Gonzalez, 2006).

Many hepatic CYP2E1 inducers can cause a rapid increase in CYP2E1 protein without affecting CYP2E1 mRNA levels; this is often achieved by an increase in CYP2E1 protein translation and/or stabilization. For example, pyridine is known to increase CYP2E1 protein levels by enhancing CYP2E1 mRNA translational efficiency (Wu et al., 1997), whereas inducers such acetone and ethanol can bind and stabilize CYP2E1 protein in vitro, resulting in decreased protein turnover and increased protein levels (Song et al., 1989; Roberts et al., 1995).

Interestingly, the mechanism of hepatic CYP2E1 induction by ethanol can be dose specific. At low ethanol concentrations CYP2E1 is induced mainly by protein stabilization, but at higher ethanol concentration (>65 mM, 0.3% ethanol by volume) CYP2E1 induction occurs via a transcriptional mechanism and/or increased CYP2E1 mRNA stability (Ronis et al., 1993).

2.3.2 Brain CYP2E1
The regulation of CYP2E1 specifically in the brain has not been extensively studied. Certain inducers of hepatic CYP2E1, such as acetone and ethanol, also induce CYP2E1 in the brain in a
region specific manner (Sánchez-Catalán et al., 2008). For example, rats treated with 3.0 g/kg of ethanol (equivalent to 2–3 drinks in humans) for seven days showed induction of CYP2E1 protein in several brain regions including the hippocampus, cerebellum and brainstem but a significant change in CYP2E1 protein levels was not observed in other regions such as the striatum and olfactory tubercle (Howard et al., 2003). When rats were treated with a daily dose of 3.0 mg/kg ethanol for 30 days, both CYP2E1 mRNA and protein levels were increased, suggesting that at this dose and duration of ethanol exposure, the mechanism of CYP2E1 induction involves increased transcription and/or mRNA stability in rats (Zhong et al., 2012).

2.5 Genetic variation in CYP2E1

The human CYP2E1 gene is located on chromosome 10. It is approximately 20 kilobase pairs long, consisting of nine exons and encoding a 493 amino acid protein (Umeno et al., 1988). Several allelic variants of the human CYP2E1 gene have been detected (http://www.cypalleles.ki.se/cyp2e1.htm, accessed on September 15, 2013). These allelic variants include single nucleotide polymorphisms (SNPs) and/or copy number variations that can result in either decreased, normal or increased CYP2E1 enzymatic activity in vitro (Neafsey et al., 2009). The most frequently studied CYP2E1 alleles are CYP2E1*5B and CYP2E1*6. The CYP2E1*5B allele contains two SNPs (-1053C>T and -1293G>C) in the 5′flanking region of the gene and is associated with increased CYP2E1 gene expression in an in vitro expression system (Hayashi et al., 1991; Watanabe et al., 1994). The CYP2E1*6 allele contains a SNP (7632T>A) in intron 6 (Uematsu et al., 1994). CYP2E1*6 was associated with decreased metabolism of chlorzoxazone in vivo (Haufroid et al., 2002); however, the impact of CYP2E1*6 on the metabolism of other CYP2E1 substrates has not been evaluated.
Since CYP2E1 is involved in the metabolic activation of a variety of toxicants and procarcinogens (Table 1), many studies have investigated the association of CYP2E1 alleles with risk for chemically mediated toxicity and cancer. CYP2E1*5B is associated with increased risk for toxicity from certain industrial solvents that are bioactivated by CYP2E1 such as vinyl chloride (Wang et al., 2010b) and n-hexane (Zhang et al., 2006). CYP2E1*6 is associated with increased genotoxicity from styrene, an industrial chemical that is bioactivated by CYP2E1 (Vodicka et al., 2001). Many studies have shown a positive association between CYP2E1*5B and various types of cancer including hepatocellular (Munaka et al., 2003), colorectal (Morita et al., 2009; Peng et al., 2013) and nasopharyngeal cancer (Hildesheim et al., 1997; Liu et al., 2007). The CYP2E1*6 allele is associated with increased risk for certain cancers, such as head and neck (Tang et al., 2010) and oral cancer (Bouchardy et al., 2000).

3. Cytochrome P450 2B6 (CYP2B6)

3.1 CYP2B enzymes in human and other species

Humans have one functional CYP2B isoform, namely CYP2B6 (http://drnelson.uthsc.edu/CytochromeP450.html). CYP2B6 is highly expressed in the liver (Figure 1) but is also constitutively expressed in the brain, kidney, lung, nasal mucosa and the gastrointestinal tract (Ding and Kaminsky, 2003; Alekxa et al., 2005; Dutheil et al., 2009; Leclerc et al., 2010). In the brain, CYP2B6 is expressed in a region-specific manner, with the highest regional expression detected in the striatum and cerebellum (Miksys et al., 2003).

Members of the CYP2B subfamily have also been identified in other mammalian species including monkeys, rats and mice (http://drnelson.uthsc.edu/CytochromeP450.html) (Table 4).


<table>
<thead>
<tr>
<th>SPECIES</th>
<th>MAJOR ISOFORMS</th>
<th>% AMINO ACID SIMILARITY RELATIVE TO HUMAN CYP2B6&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>CYP2B6</td>
<td>-</td>
</tr>
<tr>
<td>Monkey&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CYP2B6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>91</td>
</tr>
<tr>
<td>Rat</td>
<td>CYP2B1</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>CYP2B2</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>CYP2B3</td>
<td>68</td>
</tr>
<tr>
<td>Rodent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>CYP2B9</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>CYP2B10</td>
<td>78</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percent sequence similarity determined using Clustal W, http://www.genome.jp/tools/clustalw

<sup>b</sup>Rhesus monkey (macaca mulatta) was used as the reference monkey

<sup>c</sup>Also referred to as CYP2B17

Like humans, monkeys have only one functional CYP2B isoform, which is usually referred to as CYP2B6 but is occasionally called CYP2B17 (Uno et al., 2009). Rats express several CYP2B isoforms, of which CYP2B1, CYP2B2 and CYP2B3 are the major constitutively expressed enzymes (Jean et al., 1994). The major CYP2B isoforms expressed in mice are CYP2B9 and CYP2B10 (Martignoni et al., 2006).

### 3.2 CYP2B6 substrates and inhibitors

CYP2B6 substrates, while diverse, are generally non-planar, intermediate molecular weight molecules (Lewis et al., 1999). CYP2B6 metabolizes numerous compounds including therapeutic drugs, drugs of abuse, toxins and endogenous compounds (Table 5) (Mo et al., 2009).
### Table 5. Examples of CYP2B6 substrates

<table>
<thead>
<tr>
<th>EXOGENOUS</th>
<th>Toxins</th>
<th>ENDOGENOUS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drugs</strong></td>
<td><strong>Toxins</strong></td>
<td></td>
</tr>
<tr>
<td>Anesthetics</td>
<td>Anti-psychotic</td>
<td>Procacinogens</td>
</tr>
<tr>
<td>Propofol</td>
<td>Diazepam</td>
<td>Aflatoxin-B1</td>
</tr>
<tr>
<td>Ketamine</td>
<td>Anti-retrovirals</td>
<td>Dibenzanthracene</td>
</tr>
<tr>
<td>Ropivacaine</td>
<td>Efavirenz</td>
<td>NNK</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Nevirapine</td>
<td>Nitrosomorpholine</td>
</tr>
<tr>
<td>Sevoflurane</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antidepressant</strong></td>
<td>Beta-blocker</td>
<td><strong>Fatty acids</strong></td>
</tr>
<tr>
<td>Sertraline</td>
<td>Carteolol</td>
<td>Lauric acid</td>
</tr>
<tr>
<td>Bupropion</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antiepileptic</strong></td>
<td>Drugs of abuse</td>
<td><strong>Neurotransmitter</strong></td>
</tr>
<tr>
<td>Valproic acid</td>
<td>Cocaine</td>
<td>Serotonin</td>
</tr>
<tr>
<td></td>
<td>Ecstasy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nicotine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phencyclidine</td>
<td></td>
</tr>
<tr>
<td><strong>Anti-malarial</strong></td>
<td><strong>Pesticides</strong></td>
<td>Steroids</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>Drugs of abuse</td>
<td>Dihydroartemisinin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Organic Solvents</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toluene</td>
<td></td>
</tr>
<tr>
<td><strong>Antineoplastics</strong></td>
<td><strong>Procarcinogens</strong></td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Antineoplastics</td>
<td></td>
</tr>
<tr>
<td>Ifosfamide</td>
<td>Dibenzanthracene</td>
<td></td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>NNK</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitrosomorpholine</td>
<td></td>
</tr>
</tbody>
</table>

Bupropion is the most commonly used probe substrate to assess CYP2B6 activity, both *in vitro* and *in vivo* (Faucette et al., 2000; Hesse et al., 2004, Kirchheiner et al, 2003). Bupropion is an antidepressant and a non-nicotinic smoking cessation aid that is metabolized almost exclusively by CYP2B6 to its major metabolite 6-hydroxybupropion (Hesse et al., 2000).

Examples of CYP2B6 inhibitors are listed Table 6, along with their mechanism of action. Of these CYP2B6 inhibitors, ticopidine is the most potent, while N,N'N'-triethylenethiophosphoramide (ThioTEPA) is the most selective for CYP2B6 (Turpeinen et al., 2004). Ticlopidine and clopidogrel are both commonly used anti-platelet drugs, and can potentially interact with other drugs that are substrates for CYP2B6 to cause adverse drug
reactions. For example, ticlopidine decreased the \textit{in vivo} clearance of ketamine in humans, presumably by inhibiting CYP2B6-mediated ketamine metabolism (Peltoniemi et al., 2011).

\textbf{Table 6. Examples of CYP2B6 inhibitors}

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>MODE OF INHIBITION</th>
<th>\textbf{Ki in HLM (µM)}</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ticlopidine</td>
<td>Mechanism based</td>
<td>0.2-0.8</td>
<td>(Turpeinen et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Richter et al., 2004)</td>
</tr>
<tr>
<td>C8-Xanthate</td>
<td>Mechanism based</td>
<td>1</td>
<td>(Yanev et al., 1999)</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>Mechanism based</td>
<td>1.1</td>
<td>(Richter et al., 2004)</td>
</tr>
<tr>
<td>ThioTEPA</td>
<td>Mechanism based</td>
<td>2.8-3.8</td>
<td>(Turpeinen et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Richter et al., 2005)</td>
</tr>
</tbody>
</table>

\textbf{3.3 CYP2B6 regulation}

\textbf{3.3.1 Hepatic CYP2B6}

Hepatic CYP2B6 is highly inducible by several commonly used drugs, including the prototypical CYP inducers rifampicin, phenytoin, and phenobarbital (Table 7). The induction of CYP2B6 is mainly transcriptionally mediated and involves regulation by the constitutive androstane receptor (CAR; NR1I3), pregnane X receptor (PXR; NR1I2), retinoic X receptor (RXR; NR2B) and glucocorticoid receptor (NR3C1) (Mo et al., 2009).

\textbf{Table 7. Examples of hepatic CYP2B6 inducers}

<table>
<thead>
<tr>
<th>INDUCER</th>
<th>MECHANISM OF INDUCTION</th>
<th>REFERENCE</th>
</tr>
</thead>
</table>

CAR: constitutive androstane receptor; GR: glucocorticoid receptor; PXR: pregnane X receptor

In human hepatocytes phenobarbital up-regulates CYP2B6 transcription through the activation of CAR (Sueyoshi et al., 1999). Phenobarbital facilitates the nuclear translocation of CAR, possibly via a mechanism that involves dephosphorylation. Once in the nucleus, CAR forms a heterodimer with RXR and activates $CYP2B6$ gene transcription by binding the phenobarbital response enhancer module (PBREM) (Sueyoshi et al., 1999) within the $CYP2B6$ gene promoter region.

PXR mediates transactivation of the CYP2B6 PBREM in response to xenobiotics such as rifampicin (Goodwin et al., 2001). Similar to CAR, upon activation, PXR translocate to the nucleus where it can heterodimerize with RXR and bind to a xenobiotic response element (XREM) element within the promoter of the $CYP2B6$ gene (Mäkinen et al., 2002; Squires et al., 2004; Wang et al., 2003a). Many CYP2B6 inducers, such as phenobarbital and phenytoin, can activate both CAR and PXR, suggesting cross-talk between the two nuclear receptors signaling pathways resulting in co-regulation of the $CYP2B6$ gene (Wang and Negishi, 2003).

The dexamethasone-activated glucocorticoid receptor can enhance $CYP2B6$ transcription in the presence of CAR and PXR activators (Pascussi et al., 2000; Wang et al., 2003b). The glucocorticoid receptor is activated upon binding of glucocorticoids in various tissues and can regulate gene transcription by activation as well as repression (Ratman et al., 2012).
3.3.2 Brain CYP2B6

Hepatic inducers of CYP2B6, such as phenobarbital and phenytoin, can also induce CYP2B in rat brain, in a region-specific manner (Rosenbrock et al., 1999; Upadhya et al., 2002). Chronic nicotine treatment can selectively induce CYP2B mRNA and protein levels in specific regions of rat brain, without affecting hepatic CYP2B levels (Miksys et al., 2000). The induction of CYP2B by nicotine is organ-specific, suggesting that the mechanism in brain does not involve CAR or PXR, which mediate CYP2B induction in the liver.

3.4 CYP2B6 genetic variation and associated health risks

The human CYP2B6 gene is located on chromosome 19. It is approximately 27 kilobase pairs long, consisting of nine exons, and encoding a 491 amino acid protein (Yamano et al., 1989). The CYP2B6 gene is highly polymorphic; numerous SNPs are present in the promoter, introns, and exons of the CYP2B6 gene and to date, 63 different CYP2B6 alleles have been identified (http://www.cypalleles.ki.se/cyp2b6.htm, accessed September 15, 2013). Two CYP2B6 alleles that have been extensively studied are CYP2B6*5 and CYP2B6*6.

Most studies show that the CYP2B6*5 allele (SNP 459C>T) is associated with lower protein expression and activity (Lang et al., 2001; Lamba et al., 2003). The CYP2B6*6 allele is characterized by two SNPs, 516G>T and 785A>G, and is associated with lower CYP2B6 protein levels (Lang et al., 2001; Desta et al., 2007). The effects of CYP2B6*6 on enzyme function are varied, causing either increased or decreased activities depending on the substrate tested (Nakajima et al., 1996a; Hesse et al., 2004; Desta et al., 2007).

Genetic variation in CYP2B6 has been shown to impact the pharmacokinetics and efficacy of several drugs. For example, cyclophosphamide is a chemotherapeutic pro-drug that
is used in the treatment of certain cancers and autoimmune diseases; it is metabolically activated by CYP2B6 (Chang et al., 1993). The reduced function allele CYP2B6*5 has been associated with decreased activation and decreased response to cyclophosphamide in patients with lupus nephritis (Takada et al., 2004). In contrast, CYP2B6*6 is associated with increased activation and potentially increased toxic side effects from cyclophosphamide (Nakajima et al., 2007). The anti-retroviral drug efavirenz is metabolically inactivated by CYP2B6 (Ward et al., 2003). Patients possessing the CYP2B6*6 allele achieved elevated plasma levels of efavirenz after a standard dosing, and were more likely to experience neurotoxic side effects (Tsuchiya et al., 2004; Ribaudo et al., 2010).

4. Cytochrome P450 2A6 (CYP2A6)

4.1 Expression of CYP2A enzymes in humans and other animals

The human CYP2A subfamily consists of the three isoforms: CYP2A6, CYP2A7, and CYP2A13 (http://drnelson.uthsc.edu/CytochromeP450.html). CYP2A6 is highly expressed in the liver and also other tissues, such as lung, nasal mucosa and gastrointestinal tract (Ding and Kaminsky, 2003; Leclerc et al., 2010). In the human brain, low levels of CYP2A6 mRNA have been identified (Dutheil et al., 2009), but the detection of CYP2A6 protein has not been reported. CYP2A7 does not produce a functional protein (Ding et al., 1995) and CYP2A13 is mainly expressed in the respiratory tract (Su et al., 2000).

CYP2A enzymes have been identified in monkeys, rats and mice (http://drnelson.uthsc.edu/CytochromeP450.html) and are listed in Table 8. Three CYP2A isoforms have been identified in monkeys: CYP2A23, CYP2A24 and CYP2A27. Monkey CYP2A23 protein is highly homologous to human CYP2A6 and by virtue of its similar function
and regulation it is often referred to as CYP2A6. Rats have three CYP2A enzymes: CYP2A1, CYP2A2 and CYP2A3 (Martignoni et al., 2006). Mice have four CYP2A enzymes, namely CYP2A4, CYP2A5, CYP2A12 and CYP2A22 (Martignoni et al., 2006).

Table 8. CYP2A enzymes in human, monkeys, rats and mice

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>ISOFORM</th>
<th>% AMINO ACID SIMILARITY RELATIVE TO HUMAN CYP2A6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>CYP2A6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CYP2A7</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>CYP2A13</td>
<td>93</td>
</tr>
<tr>
<td>Primate</td>
<td>CYP2A6/23</td>
<td>93</td>
</tr>
<tr>
<td>Monkey</td>
<td>CYP2A24</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>CYP2A27</td>
<td>93</td>
</tr>
<tr>
<td>Rat</td>
<td>CYP2A1</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>CYP2A2</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>CYP2A3</td>
<td>85</td>
</tr>
<tr>
<td>Rodent</td>
<td>CYP2A4</td>
<td>84</td>
</tr>
<tr>
<td>Mouse</td>
<td>CYP2A5</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>CYP2A12</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>CYP2A22</td>
<td>69</td>
</tr>
</tbody>
</table>

*Percent sequence similarity determined using Clustal W, http://www.genome.jp/tools/clustalw*

bRhesus monkey (macaca mulatta) was used as the reference monkey

4.2 CYP2A6 substrates and inhibitors

The CYP2A6 enzyme participates in the metabolism of several therapeutic drugs and toxic chemicals (Table 9) (Abu-Bakar et al., 2013), and recently it was shown that bilirubin, the breakdown product of heme, is a high affinity substrate for CYP2A6 (Abu-Bakar et al., 2012).
Table 9. Examples of CYP2A6 substrates

<table>
<thead>
<tr>
<th>EXOGENOUS</th>
<th>ENDOGENOUS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drugs</strong></td>
<td><strong>Toxins</strong></td>
</tr>
<tr>
<td>Anesthetics</td>
<td>Carcinogens</td>
</tr>
<tr>
<td>Halothane</td>
<td>NNK</td>
</tr>
<tr>
<td>Antiepileptic</td>
<td>NDEA</td>
</tr>
<tr>
<td>Losigamone</td>
<td>Aflatoxin-B</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>Organic solvents</td>
</tr>
<tr>
<td>Antineoplastic</td>
<td>Chloroform</td>
</tr>
<tr>
<td>Letrozole</td>
<td>Organic solvents</td>
</tr>
<tr>
<td>Tegafur</td>
<td>Chloroform</td>
</tr>
<tr>
<td>Antiretrovirals</td>
<td>Pesticides</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>Dichlorobenzonitrile</td>
</tr>
<tr>
<td>Herbal supplements</td>
<td>Industrial chemicals</td>
</tr>
<tr>
<td>Coumarina</td>
<td>Methyl tert-butyl ether</td>
</tr>
<tr>
<td>Drugs of abuses</td>
<td>Butadiene</td>
</tr>
<tr>
<td>Nicotinea</td>
<td>MOCA d</td>
</tr>
</tbody>
</table>

a Also used clinically  
bNNK: 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanone  
cNDEA: N-Nitrosodiethylamine  
dMOCA: 4,4-methylenebis (2-chloroaniline)

Among the compounds listed in Table 9, coumarin and nicotine are most commonly used as probe substrates to measure CYP2A6 activity. Coumarin is a natural plant alkaloid present in a number of herbal medicinal products. It is specifically metabolized to 7-hydroxycoumarin by CYP2A6; the rate of this reaction is used to assess CYP2A6 activity both in vitro and in vivo (Pelkonen et al., 2000). Following oral administration of coumarin, approximately 95-98% is rapidly excreted as 7-hydroxycoumarin glucuronide (Ritschel et al., 1977). The rapid elimination of coumarin makes it an excellent probe substrate for determining the presence or absence of CYP2A6 activity in vivo, but is a poor indicator for discriminating among a narrow range of CYP2A6 activities in vivo.
In humans, the metabolism of nicotine to cotinine is primarily (70-80%) mediated by CYP2A6, and the subsequent conversion of cotinine to trans-3-hydroxycotinine is exclusively catalyzed by CYP2A6 (Nakajima et al., 1996a; Messina et al., 1997). The trans-3-hydroxycotinine/cotinine ratio can be used to phenotype CYP2A6 activity in smokers and in non-smokers after oral nicotine dosing (Dempsey et al., 2004).

Examples of compounds that are known to inhibit CYP2A6 are listed in Table 10, along with their mechanism of action. Of these inhibitors, tranylcypromine (a monoamine oxidase inhibitor) and 8-methoxypsoralen (a treatment for cutaneous psoriasis) have been used to suppress CYP2A6 function in vivo (Tyndale and Sellers, 2001; Sellers et al., 2003).

Table 10. Examples of CYP2A6 inhibitors

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>MODE OF INHIBITION</th>
<th>Ki in HLM (µM)</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tranylcypromine</td>
<td>Competitive</td>
<td>0.08-0.2</td>
<td>(Taavitsainen et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Draper et al., 1997)</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>Competitive</td>
<td>0.2</td>
<td>(Higashi et al., 2007b)</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>Competitive</td>
<td>1</td>
<td>(Kinonen et al., 1995)</td>
</tr>
<tr>
<td>8-Methoxypsoralen</td>
<td>Competitive/Mechanism-based</td>
<td>0.2-8</td>
<td>(Draper et al., 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Koenigs et al., 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Damaj et al., 2007)</td>
</tr>
</tbody>
</table>

4.3 Regulation of hepatic CYP2A6

The regulation of hepatic CYP2A6 involves both transcriptional and post-transcriptional mechanisms. Inducers of CYP2A6 include a variety of drugs, toxins and pathophysiological states (Table 11).
Table 11. Examples of CYP2A6 inducers

<table>
<thead>
<tr>
<th>INDUCER</th>
<th>MECHANISM OF INDUCTION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td>CAR/PXR</td>
<td>(Donato et al., 2000)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>CAR/PXR</td>
<td>(Rae et al., 2001)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>GR and HNF4-α</td>
<td>(Onica et al., 2008)</td>
</tr>
<tr>
<td>Ethinyl estradiol</td>
<td>ERα</td>
<td>(Higashi et al., 2007a)</td>
</tr>
<tr>
<td>Pyrazole</td>
<td>mRNA stabilization$^a$</td>
<td>(Donato et al., 2000)</td>
</tr>
</tbody>
</table>

$^a$Assuming a similar mechanism as the induction of CYP2A5 by pyrazole

The prototypical CYP inducers phenobarbital and rifampicin can induce CYP2A6 in human primary hepatocytes (Donato et al., 2000; Rae et al., 2001). As previously discussed, rifampicin is an activator of PXR (Sueyoshi et al., 1999) and phenobarbital can activate both PXR and CAR (Goodwin et al., 2001). Upon activation, CAR and PXR can dimerize with RXR and bind to direct repeat 4 (DR4)-like elements at −6698, −5476, and −4618 on the CYP2A6 gene (Itoh et al., 2006). PXR can interact with peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) at the DR4-like response elements at -5476 and -4618 on CYP2A6 to increase transcription (Itoh et al., 2006).

CYP2A6 is under the regulation of estrogen receptor alpha (ERα; NR3A1) (Higashi et al., 2007a). This is reflected by increased nicotine and cotinine metabolism in females taking estrogen-containing oral contraceptives (Benowitz et al., 2006a). Induction of CYP2A6 by estrogen is also consistent with the faster clearance of nicotine in females compared to males (Benowitz et al., 2006a), and during pregnancy (Dempsey et al., 2002).

In human hepatocytes, dexamethasone can induce CYP2A6 expression via a mechanism that involves the interaction of the glucocorticoid receptor and hepatic nuclear factor 4-α (HNF4-
α; NR2A1) at the HNF4-α response element located within the CYP2A6 promoter region (Onica et al., 2008). In addition, the promoter region of the CYP2A6 gene contains response elements for HNF-1α, CCAAT-box/enhancer binding protein α and β (C/EBPα, C/EBPβ), octamer transcription factor-1 (Oct-1) and Nuclear factor-E2 p45-related factor (Nrf2), which are important in regulating the basal expression of CYP2A6 (Pitarque et al., 2005; Yokota et al., 2011).

Post-transcriptional regulation of CYP2A6 is mediated by the interaction of the RNA-binding protein heterogeneous nuclear ribonucleoprotein (hnRNPA1) with the 3’-untranslated region of CYP2A6 mRNA (Christian, 2004). It has been proposed that this interaction between hnRNPA1 and CYP2A6 mRNA results in the stabilization CYP2A6 mRNA (Christian, 2004). The post-transcriptional regulation of mouse CYP2A5 by hnRNPA1 has been extensively studied. Pyrazole, which is a potent inducer of hepatic CYP2A5/6 protein and mRNA, was shown to increase cytoplasmic levels of hnRNPA1 in mouse hepatocytes (Raffalli-Mathieu et al., 2002). Also, the overexpression of hnRNPA1 in mouse primary hepatocytes caused an accumulation of CYP2A5 mRNA (Raffalli-Mathieu et al., 2002). Furthermore, pyrazole treatment induces binding of hRNP1 to the 3’ untranslated region of CYP2A5 and is association with CYP2A5 mRNA stabilization (CYP2A5 mRNA half-life is approximately 1.5h in untreated mice and 6h in pyrazole-treated mice) and elongation of the CYP2A5 mRNA poly(A) tail (Aida and Negishi, 1991; Geneste et al., 1996). Collectively, these findings suggest that hnRPA1 is involved in the post-transcriptional regulation of CYP2A5.
4.4 CYP2A6 genetic variation and associated health risks

The human CYP2A6 gene is located on chromosome 19. It is approximately 6 kilobase pairs long, consisting of nine exons, and encoding a 494 amino acid protein (Fernandez-Salguero et al., 1995a). The CYP2A6 gene is highly polymorphic; it can exist as a deleted or duplicated gene and can contain gene conversions, nucleotide deletions, nucleotide insertions and SNPs (Mwenifumbo and Tyndale, 2007). There are over 50 allelic variants of the CYP2A6 gene (http://www.imm.ki.se/CYPalleles/cyp2a6.htm, accessed September 15, 2013), many of which have been characterized in vitro and/or in vivo and result in absent, reduced, increased, or normal enzyme activity (Mwenifumbo and Tyndale, 2007).

Individuals can be classified as normal, intermediate or slow CYP2A6 metabolizers based on their CYP2A6 genotype (Benowitz et al., 2006b). CYP2A6 normal metabolizers consist of individuals who do not possess any known reduced or loss-of-function alleles (i.e. a wild-type CYP2A6*1/*1 genotype). The intermediate metabolizers are individuals possessing one copy of a known CYP2A6 reduced function allele (e.g., CYP2A6*9 or CYP2A6*12), whose hypothesized CYP2A6 activity would be approximately 75% of wild-type levels (Oscarson et al., 2002; Benowitz et al., 2006b; Ho et al., 2009). The slow metabolizer group contains individuals who possess one or more copies of known CYP2A6 loss-of-function alleles (CYP2A6*2, *4, *7, *9, *10, *17, *35), two copies of known reduced function CYP2A6 alleles or a combination of reduced and loss-of-function alleles; these individuals are hypothesized to have less than 50% of wild-type CYP2A6 activity (Malaiyandi et al., 2006; Benowitz et al., 2006b; Ho et al., 2009; Lerman et al., 2010). CYP2A6 intermediate and slow metabolizer groups are often combined to generate a reduced metabolizer group; reduced metabolizers possess at least one copy of a known CYP2A6 reduced function allele (Wassenaar et al., 2011; Zhu et al., 2013).
CYP2A6 is the main enzyme involved in the metabolism of nicotine and genetic variation in CYP2A6 has been associated with differences in nicotine pharmacokinetics and smoking behaviours. Compared to CYP2A6 normal metabolizers, reduced metabolizers have slower nicotine clearance (Dempsey et al., 2004), smoke less cigarettes per day (Schoedel et al., 2004; Johnstone et al., 2006; Minematsu et al., 2006), smoke for shorter durations (Schoedel et al., 2004) and have higher cessation success rates (Patterson et al., 2008; Ho et al., 2009), particularly with transdermal nicotine patch therapy (Schnoll et al., 2009; Lerman et al., 2010). CYP2A6 also metabolically activates several pro-carcinogens present in cigarette smoke, such as the potent pulmonary carcinogen NNK (Table 9) (Tiano et al., 1994). Compared to CYP2A6 normal metabolizers, slow metabolizers are less likely to develop lung cancer (Wassenaar et al., 2011; Liu et al., 2013). Genetic variation in CYP2A6 also contributes to variability in treatment response to the chemotherapeutic pro-drug tegafur (Park et al., 2011). To exert its anti-tumour effect tegafur must be metabolically converted by CYP2A6 to the active metabolite 5-fluorouracil (Ikeda et al., 2000). Among patients with gastric cancer receiving tegafur treatment, the CYP2A6 slow metabolizers had decreased 5-fluorouracil plasma levels and decreased overall survival (Park et al., 2011).

5. The effects of alcohol use and smoking on cytochromes P450

Ethanol and nicotine are known or hypothesized to regulate the expression of CYP2E1, CYP2B6 and CYP2A6 in liver and/or brain. An increase or decrease in the expression of these CYPs due to ethanol and/or nicotine exposure is a concern, given the widespread use of alcohol and tobacco, and the potential adverse consequences of altered CYP activity. In this chapter, some relevant features of ethanol and nicotine will be reviewed including: the prevalence of alcohol
and tobacco use and co-use, the known effects of ethanol and nicotine on CYP expression and activity in liver and/or brain, and a brief overview of ethanol and nicotine pharmacokinetics.

5.1 Prevalence of alcohol and tobacco use

5.1.1 Alcohol use

Based on survey data from 2005, it was estimated that over 40% of the world’s adult population (≥ 15 years old) consumes alcohol and 6% of adults worldwide are heavy regular drinkers (i.e., men drinking at least 5 standard drinks per day and women drinking at least 3 standard drinks/day) (Shield et al., 2013). The levels of alcohol consumption and patterns of drinking vary widely across regions of the world (Figure 2). For example, in South East Asia, less than 20% of adults consume alcohol and 2% of adults are heavy regular drinkers, whereas in Western Europe, almost 90% of adults consume alcohol and 12% of adults are heavy regular drinkers (Shield et al., 2013). Adult per capita alcohol consumption reflects the amount of pure ethanol consumed by a population in a given year and is considered to be an accurate and reliable indicator of a population's alcohol consumption (Gmel and Rehm, 2004).
**Figure 2. Worldwide per capita alcohol consumption for adults (≥ 15 years old).**
Per capita alcohol consumption in litres is based on survey data taken in 2005. This figure is adapted and modified from the (World Health Organization, 2011).

### 5.1.2 Tobacco use

Most of the tobacco that is consumed throughout the world is in the form of cigarettes (Giovino et al., 2012); however, tobacco is also smoked in other products (e.g. cigars and water pipes) and many smokeless tobacco products exist (e.g. chewing tobacco and snuff). Based on a 2010 survey that collected tobacco use data from 16 countries, it was estimated that approximately 30% of the world’s adult population (≥ 15 years old) are tobacco users (Giovino et al., 2012). The prevalence of tobacco use varies considerably among different countries (Figure 3). For example, in Russia 40% of the adult population are tobacco users, while in Mexico 16% of the population are tobacco users (Giovino et al., 2012).
Figure 3. Proportion of the adults (≥ 15 years old) who use tobacco categorized by gender and by country.
Tobacco use was defined as tobacco smoking or using smokeless tobacco on a daily or less than daily basis. This figure is based on data from (Giovino et al., 2012).

5.1.3 Alcohol and tobacco co-use

Alcohol consumption and tobacco use are closely linked behaviours. People who drink alcohol are more likely to smoke, and people who drink larger amounts of alcohol tend to smoke more cigarettes (De Leon et al., 2007). In fact, the prevalence of smoking among alcoholics has been estimated to be 50-80% (depending on the study), significantly higher than the prevalence of smoking in the general population (Figure 4) (Ellingstad et al., 1999; Lasser et al., 2000; Grant et
al., 2004). Similarly, smokers are more likely to consume alcohol than non-smokers, and smokers who are dependent on nicotine have a four-fold greater risk of becoming alcohol dependent compared to non-smokers (Grant et al., 2004). Shown in Figure 4 are the results from a study conducted in 2001-2002 to determine the prevalence of alcohol and tobacco co-use in the United States, demonstrating how tobacco use is related to differing levels of alcohol consumption (Falk et al., 2006). These results emphasize the difficulties in studying the effects of heavy alcohol use or smoking in isolation. It was estimated that 22% of adults in the United States co-use alcohol and tobacco (Falk et al., 2006).

![Prevalence of daily tobacco use (Percent +/- SE)](image)

**Figure 4. Prevalence of tobacco grouped by alcohol consumption characteristics and alcohol dependence.**
Data obtained from a 2005 survey of adults ≥18 years old in the United States. Figure is based on data from (Falk et al., 2006).

### 5.2 The effect of alcohol use and smoking on hepatic cytochromes P450

Certain CYPs can be induced by ethanol and nicotine. Changes in CYPs can lead to changes in xenobiotic clearance and biotransformation, potentially having clinically important consequences. In the preceding section, current knowledge regarding the impact of ethanol and
nicotine on CYP2E1, CYP2A6 and CYP2B6 in the liver and/or brain will be reviewed.

5.2.1 Hepatic CYP2E1

Ethanol is an established inducer of hepatic CYP2E1. In humans, daily ingestion of 40 g of ethanol (about 3 standard drinks) for one week induced in vivo chlorzoxazone clearance, a measure of CYP2E1 activity (Oneta et al., 2002; Liangpunsakul et al., 2005). In addition, alcoholics can have a 4-10 fold increase in hepatic CYP2E1 protein levels compared to non-alcoholics (Tsutsumi et al., 1989; Lieber, 1999). Ethanol treatment also induced hepatic CYP2E1 levels in monkeys, rats and mice (Forkert et al., 1991; Howard et al., 2001; Ivester et al., 2007).

Smokers have higher hepatic CYP2E1 activity compared to non-smokers (Benowitz et al., 2003), which is presumably due to the effects of nicotine based on studies showing that nicotine can induce hepatic CYP2E1 protein levels in monkeys and rats (Micu et al., 2003; Lee et al., 2006c).

Since ethanol and nicotine can independently induce CYP2E1, it is possible that combined exposure to both drugs can results in additive or even synergistic effects on CYP2E1 protein levels and activity. Rats exposed to both ethanol and nicotine had significantly greater levels of hepatic CYP2E1 protein compared with rats exposed to either drug alone (Yue et al., 2009). This enhancement of CYP2E1 protein was partially attributed to a nicotine-stimulated increase in ethanol consumption. There was no indication as to how the observed increases in CYP2E1 protein levels would affect in vitro and in vivo CYP2E1 activity.
5.2.2 Hepatic CYP2B6

Smoking has no effect on hepatic CYP2B6 protein levels or activity in humans (Hesse et al., 2004). Consistent with this, nicotine treatment has no effect on hepatic CYP2B6 protein levels in monkeys (Lee et al., 2006b). However, hepatic CYP2B6 expression and activity are higher in drinkers (consuming >14 drinks per week) compared to individuals who do not consume alcohol (Hesse et al., 2004). In rats, chronic alcohol administration induces hepatic CYP2B1/2 (Schoedel et al., 2001). The effect of ethanol on hepatic CYP2B6 protein levels or activity has not been studied in monkeys.

5.2.3 Hepatic CYP2A6

Smokers have decreased nicotine metabolism compared to non-smokers, which is hypothesized to be caused by the down regulation of CYP2A6 by nicotine (Benowitz and Jacob, 2000). Chronic nicotine treatment downregulates hepatic CYP2A6 in monkeys and results in decreased \textit{in vitro} nicotine metabolism (Schoedel et al., 2003). Conflicting results have been published with respect to the effect of alcohol consumption on hepatic CYP2A6 activity in humans (Niemelä et al., 2000; Mwenifumbo et al., 2007). In rats, CYP2A5 (homologous to human CYP2A6) activity was not affected by ethanol treatment (Honkakoski et al., 1988). The effect of ethanol on hepatic CYP2A6 has not been studied in monkeys.

5.4 Brain CYP2E1 and CYP2B6 in alcoholics and smokers

Alcoholics have higher brain levels of CYP2E1 and CYP2B6 compared to non-alcoholics and it is not known whether this is due to ethanol exposure, or other variables associated with alcoholism such as tobacco use (Howard et al., 2003; Miksys et al., 2003). Compared to non-
alcoholics, alcoholics had higher CYP2E1 protein levels in the hippocampus and cerebellum (Howard et al., 2003), as well as higher CYP2B6 in the hippocampus, caudate and putamen (Miksys and Tyndale, 2009). To test the hypothesis that ethanol induces brain levels of CYP2E1 and CYP2B, the expression of CYP2E1 and CYP2B1 was investigated in rats chronically treated with 3.0 g/kg ethanol (Schoedel et al., 2001; Howard et al., 2003). Ethanol treatment induced CYP2E1 in the olfactory bulbs, frontal cortex, hippocampus, and cerebellum of rat brain (Howard et al., 2003). However, ethanol treatment did not affect CYP2B1 protein levels in any of the rat brain regions that were assessed (Schoedel et al., 2001). Therefore, the pattern of expression of ethanol-induced CYP2E1 in rat brain differs from that found in alcoholics, and furthermore, ethanol had no effect on CYP2B1 levels. This discrepancy may be explained by species differences (primate vs. rodent) or differences in ethanol dose/route of administration (self-administration vs. gavage). To clarify this, the effect of ethanol on brain CYP2E1 and CYP2B6 should be studied in primates and ethanol should be delivered via self-administration.

Smokers have higher brain levels of CYP2E1 and CYP2B6 compared to non-smokers and it is not known whether this is due to nicotine, other components of smoke and/or other variables associated with smoking such as alcohol consumption (Howard et al., 2003; Miksys et al., 2003). Compared to non-smokers, smokers had higher CYP2E1 in the frontal cortex, hippocampus and cerebellum, whereas CYP2B6 was higher in the hippocampus, caudate, putamen and cerebellum. To test the hypothesis that nicotine induces brain CYP2E1 and CYP2B6, expression of both enzymes was investigated in monkeys treated with 0.3 g/kg (bid) nicotine chronically (Joshi and Tyndale, 2006b; Lee et al., 2006b). Induction of CYP2E1 was observed in the frontal cortex and cerebellum (Joshi and Tyndale, 2006a), whereas induction of CYP2B6 was only seen in the frontal cortex (Lee et al., 2006b). Therefore, nicotine treatment in
monkeys resulted in the induction CYP2E1 and CYP2B6 in fewer brain regions than what had been observed in the human smokers. This may be because other factors contribute to the higher brain levels of CYP2E1 and CYP2B6 in smokers. Alternatively, the dose of nicotine used in the monkey study may not have been high enough to see the effect observed in human smokers.

6. Ethanol pharmacokinetics

6.1 Absorption and distribution
Ethanol is a small water-soluble molecule that can passively diffuse across membranes. When consumed orally, approximately 10-20% of an ethanol dose is absorbed by the stomach and the remainder is absorbed through the small intestine (Lester and Greenberg, 1951). Due to its large surface area and rich blood supply, the small intestine can absorb ethanol more rapidly compared to the stomach. The rate of ethanol absorption can vary significantly between individuals and within the same individual at different times (Fraser et al., 1995). An important factor that reduces the rate of ethanol absorption is the presence of food in the stomach before or shortly after ethanol ingestion. The ingestion of food with ethanol prolongs the retention of ethanol in the stomach, and since the absorption of ethanol is much slower in the stomach compared to the small intestine, this delays and lowers the peak ethanol concentration (Sedman et al., 1976). For example, an ethanol dose of 0.8 g/kg produced a mean peak blood ethanol concentration of 22.6 mM (~0.10% ethanol by volume) in men who were in a fasting state; however, when the same dose of ethanol was given to the same men after a meal the resulting peak blood ethanol concentration was 35% lower, 14.6 mM (~0.07% ethanol by volume) (Jones and Jönsson, 1994). Furthermore, the mean time to reach peak blood ethanol concentration was also delayed by ~2 hrs when ethanol was consumed with food (Jones and Jönsson, 1994).
The bioavailability of orally consumed alcohol is reduced by first pass metabolism in the gastric mucosa and liver (Levitt et al., 1997). On average, approximately 20% of an ethanol dose is removed during the first pass effect; however, this value can vary depending the rate of ethanol absorption (i.e. the first pass effect is maximized when absorption is slow) (Holford, 1987). Ethanol that enters the systemic circulation is rapidly distributed through the tissues. Ethanol does not bind to plasma proteins and is completely soluble in water, and thus has a similar volume of distribution as total body water (~ 0.6-0.7 L/kg) (Cowan et al., 1996). Initially ethanol is preferentially distributed to tissues that are high in water content (e.g. skeletal muscles and brain).

6.2 Metabolism and elimination

Only a small proportion of the absorbed ethanol (2-5%) is excreted unchanged in the urine (Jones, 1990). The majority of ethanol is eliminated via oxidation by alcohol dehydrogenase (ADH) in the liver (Lands, 1998) (Figure 5). ADH converts ethanol to acetaldehyde, a reactive molecule that is rapidly oxidized by aldehyde dehydrogenase (ALDH) to acetate. Acetate is then further metabolized to carbon dioxide and water.

CYP2E1 also contributes to the metabolism of ethanol (Matsumoto et al., 1996) (Figure 5). Compared to ADH, CYP2E1 has a lower affinity for ethanol (Km ~10 mM, about 10 fold higher than ADH), and therefore at low blood ethanol concentrations (< 5 mM; 0.02% blood ethanol by volume) the contribution of CYP2E1 to ethanol clearance is limited (Keiding et al., 1983; Lands, 1998). However, at higher blood ethanol concentrations CYP2E1 may contribute to up to 40% of ethanol clearance (Matsumoto et al., 1996). Unlike ADH, CYP2E1 can be greatly induced after chronic ethanol consumption (Tsutsumi et al., 1989; Vidal et al., 1990).
Therefore, in chronic heavy alcohol drinkers CYP2E1 may play an even greater role in ethanol metabolism. A third oxidative pathway for the oxidative metabolism of ethanol is mediated by catalase (Figure 5). However, under physiological conditions, catalase appears to play no major role in systemic ethanol metabolism (Matsumoto and Fukui, 2002).

**Figure 5. Schematic showing pathways for ethanol metabolism.**
See text for a description of enzymes and reactions contributing to ethanol metabolism.

At blood ethanol concentrations greater than 5 mM (~0.02% blood ethanol by volume), which can be achieve by consuming a standard alcoholic beverage, ADH enzymes are saturated and the elimination of ethanol is concentration independent (i.e. zero order elimination) (Mumenthaler et al., 1999). The average rate of ethanol elimination can vary significantly between individuals (due to body composition, gender, genetics, alcohol use history) and ranges from 3.0 - 4.8 mM/h (~0.014-0.022 % blood ethanol by volume/h) (Lands, 1998).

7. **Nicotine pharmacokinetics**

7.1 **Nicotine absorption and distribution**

The amount of nicotine that enters the systemic circulation is highly dependent on the route of
administration. Cigarettes contain approximately 10-14 mg of nicotine (Kozlowski et al., 1998), most of which (~75%) is lost during smoking due to side stream smoke and retention of nicotine in the cigarette butt (Armitage et al., 1975). Once tobacco smoke reaches the alveoli of the lungs, about 80-90% of nicotine is rapidly absorbed (Armitage et al., 1975). On average, about 1 mg (range 0.3-2 mg) of nicotine is absorbed systemically during smoking (Benowitz and Jacob, 1984; Gori and Lynch, 1985). After a puff, high levels of nicotine reach the brain within 10-20 seconds (Benowitz, 1990). This rapid rise in nicotine levels makes smoking the most reinforcing route of nicotine administration (Benowitz, 1990). The process of smoking allows the smoker to control nicotine brain levels, and the related effects, by adjusting puff volume, depth of inhalation, rate of puffing and intensity of puffing (Scherer, 1999). The absorption of nicotine from all nicotine replacement therapies is slower compared to smoking. The more gradual increase in nicotine levels produced by nicotine replacement therapy results in their lower abuse liability (Henningfield and Keenan, 1993).

Nicotine is distributed extensively to the body tissues with an average steady state volume of distribution of 2.6 L/kg (range 2.2-3.3 L/kg) (Benowitz et al., 1991). In the blood, at a pH of 7.4, nicotine is 69% ionized and 31% unionized and the proportion of nicotine binding to plasma proteins is less than 5% (Benowitz et al., 1982). The organs with the highest affinity for nicotine are the liver, kidney and spleen, whereas adipose tissue has the lowest affinity (Urakawa et al., 1994).

7.2 Metabolism and elimination

Only 8-10% of absorbed nicotine is recovered unchanged in the urine of smokers (Benowitz et al., 1994) (Figure 7). Nicotine is extensively metabolized, predominantly in the liver. The major
pathways contributing to nicotine metabolism are shown in Figure 6.

**Figure 6. Nicotine and its metabolites recovered from human urine over 24hrs following cigarette consumption.**
Circled in purple are the urinary metabolites and the percent of systemic dose of nicotine accounted for by the specific metabolite. This figure is adapted and modified from (Hukkanen et al., 2005).

Approximately 70-80% of nicotine is metabolized to cotinine in a reaction that occurs in two sequential steps (Benowitz and Jacob 1994). The first step involves the production of an intermediate ion known as nicotine-$\Delta^1'(5')$-iminium ion (Murphy, 1973). CYP2A6 is the main
enzyme catalyzing the formation of nicotine-Δ1’(5’)-iminium ion (80-90%) with a minor (10-20%) role of CYP2B6 (Nakajima et al., 1996b; Messina et al., 1997). Individuals with genetically inactive CYP2A6 produce little or no cotinine (Benowitz et al., 2001). The second step involves the rapid conversion of nicotine-Δ’(5’)-iminium ion to cotinine by aldehyde oxidase (Brandänge and Lindblom, 1979).

Although the majority of nicotine is metabolized to cotinine, only 10-15% of the nicotine absorbed by smokers appears in urine as unchanged cotinine (Benowitz et al., 1994). This is because cotinine is also metabolized to a number of compounds, with the majority undergoing oxidation to trans-3-hydroxycotinine (3HC) (Bowman and McKennis, 1962; Neurath et al., 1987). 3HC is produced from cotinine via a reaction that is thought to be mediated exclusively by CYP2A6 (Nakajima et al., 1996a). 3HC is the most abundant nicotine metabolite found in the urine of most smokers, representing on average 33-40% of the nicotine dose (Benowitz et al., 1994).

Nicotine, cotinine and 3HC undergo substantial glucuronidation whereby glucuronic acid is added to the substrate, making the conjugated compounds more water-soluble and readily excreted. This reaction is mediated by uridine diphosphate-glucuronosyltransferases (UGTs). Nicotine and cotinine undergo N-glucuronidation by UGT2B10 (Chen et al., 2007; Kaivosaaari et al., 2007), while trans-3’-hydroxycotinine undergoes O-glucuronidation by UGT2B17 (Chen et al., 2012). The glucuronide conjugates of nicotine, cotinine, and trans-3’-hydroxycotinine account for approximately 3-5%, 12-17%, and 7-9% of the nicotine dose, respectively (Benowitz et al., 1994). Other minor metabolites of nicotine and cotinine include nicotine N-oxide (4-7%), cotinine N-oxide (2-5%), nornicotine (0.4-0.8%), and norcotinine (1-2%) (Benowitz et al., 1994). Nicotine N-oxide formation is catalyzed by flavin-containing monooxygenase 3 (FMO3).
(Cashman et al., 1992), while a CYP-mediated reaction forms cotinine N-oxide (Gorrod, 1977). Nornicotine and norcotinine can be produced by CYP2A6, CYP2A13, and CYP2B6 \textit{in vitro} (Murphy et al., 2005; Yamanaka et al., 2005).

Nicotine and its metabolites are mainly excreted in the urine; the excretion of nicotine via the feces, sweat and bile is negligible (Hukkanen et al., 2005). The renal clearance of nicotine averages 0.035-0.090 L/min (Benowitz et al., 1994).

8. The use of African green monkeys to model the regulation of CYPs by ethanol and nicotine

8.1 The use of primates for pharmacokinetic studies

Primates can be divided into four groups, including humans, apes (i.e., gibbons, orangutans, gorillas, chimpanzees), monkeys and small animals known as prosimians (Preuss, 2012) (Figure 8).

![Phylogenetic Tree](preuss2012)

**Figure 7. Phylogenetic tree showing the evolutionary relationship between primates.** This tree only represents a branching pattern and its branch spans do not represent time or relative amount of character change. Figure was modified from (Preuss, 2012)
Monkeys can be further divided into old world (e.g. African green monkey, rhesus and cynomolgus monkey) and new world monkeys (e.g. squirrel monkey and marmoset) (Preuss, 2012) (Figure 7).

Nonhuman primates are routinely used for in vivo pharmacokinetic studies to predict the disposition of drugs and other chemicals in humans. Although rats can also be used for this purpose, numerous studies have shown that optimum prediction of human disposition to xenobiotics is achieved from preclinical pharmacokinetic data from nonhuman primates (Ward et al., 2005; Evans et al., 2006; Tang et al., 2007). The African green monkey (AGM) (Chlorocebus sabeus), also referred to as the vervet monkey, has been validated and utilized as a model to predict human in vivo pharmacokinetics, particularly CYP-mediated metabolism and other clearance processes (Schoedel et al., 2003; Lee et al., 2006c; Ward et al., 2008).

8.2 History and characteristics of African green monkeys

AGMs inhabit most regions of sub-Saharan Africa and are the most populous of all the African nonhuman primates (Wolfheim, 1983). During the 17th century, a small number of AGMs were brought to the Caribbean islands of St. Kitts (McGuire, 1974). There they established feral populations that have expanded enormously over ~80-100 generations and currently tens of thousands of monkeys inhabit the island (Jasinska et al., 2012). Since the St. Kitts AGMs emerged from a small founder population, it is expected that genetic variation is reduced compared to AGM populations in Africa.

Research colonies have been established in St. Kitts, and AGMs have been utilized for a wide range of investigations (Lemere et al., 2006; Lee et al., 2006c; Redmond et al., 2008); most of the AGMs utilized in North America for biomedical investigations are descendants from the
Caribbean population. AGMs from St. Kitts have served as the founders of large research colonies in the US, for example at the Vervet Research Colony at University of California, Los Angeles (Jasinska et al., 2012).

8.3 Comparison CYP structure, expression, activity and regulation in AGMs and humans

8.3.1 CYP2E1

AGM CYP2E1 has not been sequenced, but is expected to have a similar amino acid sequence to that of human CYP2E1. This assumption is based on CYP2E1 from other old world monkeys, such as the cynomolgus monkey (Macaca fascicularis) and the rhesus monkey (Macaca mulatta), which share >90% amino acid sequence identity with human CYP2E1. Similar to humans, AGM CYP2E1 is highly expressed in the centrolobular region of the liver, and is also expressed at lower levels in extra-hepatic organs such as the brain (Joshi and Tyndale, 2006b). Chlorzoxazone is selectively hydroxylated by CYP2E1 in both AGMs and humans, and is a probe drug for CYP2E1 activity in both species (Ernstgard et al., 2004; Lee et al., 2006c). A $K_m$ of 95.4 $\mu$M and a $V_{max}$ of 3.5 pmol/min/mg for chlorzoxazone metabolism in AGM hepatic microsomes (Lee et al., 2006c) is comparable to the $K_m$ ranging from 77-149 $\mu$M and $V_{max}$ ranging from 1.5-3.2 pmol/min/mg in human hepatic microsomes (Court et al., 1997). The induction of hepatic CYP2E1 by ethanol and nicotine has been demonstrated in both cynomolgus monkeys and AGMs (Lee et al., 2006c; Ivester et al., 2007), which is consistent with the increase in CYP2E1 activity observed with human alcohol consumption and smoking (Oneta et al., 2002; Benowitz et al., 2003).
8.3.2 CYP2B6

AGM CYP2B6 protein has been sequenced (accession number Q7M3F2, GI: 75053416) and shares 91% amino acid homology with human CYP2B6. Similar to humans, AGM CYP2B6 is abundant in the liver and is also expressed at lower levels in the brain (Lee et al., 2006b). The regional pattern of CYP2B6 expression in AGM brain parallels the pattern of expression detected in human brain (Figure 8) (Lee et al., 2006b). The substrate specificity of AGM CYP2B6 is expected to be similar to that of human CYP2B6. This assumption is based on studies showing that CYP2B6 from human and cynomolgus monkey exhibit similar kinetic profiles for the oxidation of 7-ethoxy-4-(trifluoromethyl) coumarin, bupropion and efavirenz (Mayumi et al., 2013). Human and monkey CYP2B6 are both substantially induced by phenobarbital (Jao et al., 1972; Lee et al., 2006a). Also, the nicotine-treated AGMs show induction of brain CYP2B6, consistent with the higher levels of CYP2B6 detected in human smokers (Lee et al., 2006b).

![Figure 8. Relative CYP2B6 protein levels in different regions of AGM and human brain.](image)

Mean CYP2B6 protein levels in brain regions from untreated AGM correlated to mean levels in non-smoker non-alcoholic humans. Figure was adapted and modified from (Lee et al., 2006b).
8.3.3 CYP2A6

Similarity between AGM and human CYP2A6 is expected to be fairly high (based on >90% amino acid sequence identity between human and rhesus monkey CYP2A6); however, the AGM CYP2A6 gene or protein has not been sequenced. As with humans, AGM CYP2A protein is abundant in the liver (Schoedel et al., 2003), but has not been reported in the brain. Coumarin 7-hydroxylation is a specific probe for human CYP2A6 activity (Pelkonen et al., 2000). *In vitro*, this pathway is similar between humans and AGMs ($K_m$ of 2.1 μM and $V_{max}$ of 0.79 nmol/mg of protein/min in humans compared with $K_m$ of 2.7 μM and $V_{max}$ of 0.52 nmol/mg of protein/min in AGM) (Li et al., 1997). The downregulation of CYP2A6 by nicotine has been demonstrated in AGM (Schoedel et al., 2003), which is consistent with the decrease in nicotine clearance in human smokers (Benowitz and Jacob, 2000).

8.4 Ethanol self-administration and pharmacokinetics in African green monkeys

Most AGMs will readily self-administer ethanol, at levels comparable to human consumption, with no behavioural training or dietary deprivation (Ervin et al., 1990). Out of 1000 monkeys screened from a feral population, approximately 85% voluntarily drank ethanol diluted in sucrose solution, 15% were heavy drinkers (consuming >5 g ethanol/kg/day) and 5% drank repeatedly to the point of coma (Palmour et al., 1997). This distribution is similar to patterns of alcohol consumption seen in certain human populations (section 5.1.1). AGM alcohol consumption behaviour is strikingly different from most rodents, which must be trained to self-administer ethanol and only consume modest amounts. Most AGMs prefer their ethanol in a
sweetened fluid (e.g. in sucrose solution); however, heavy consumers will drink unsweetened alcohol diluted in water (Palmour et al., 1997).

Monkeys have relatively similar ethanol absorption and metabolism kinetics compared to humans (Green et al., 1999). However, the overall rate of ethanol elimination is faster in old world monkeys compared to humans (Zorzano and Herrera, 1990). AGMs eliminate ethanol at a rate of 8.4 – 13.2 mM/h (~0.04 – 0.06% ethanol by volume/h) (Ervin et al., 1990; Grant and Bennett, 2003), whereas humans eliminate ethanol at a rate of 3.0-4.8 mM/h (~ 0.01-0.02% ethanol by volume/h) (Lands, 1998). The reason for faster ethanol elimination in monkeys is not known. When given the same g/kg ethanol dose, monkeys will achieve lower peak blood ethanol concentrations compared to humans (Zorzano and Herrera, 1990). For this reason, caution is warranted when comparing levels of ethanol intake between AGM and humans (Grant and Bennett, 2003); comparisons based on blood ethanol concentration (and not based on g/kg) are often used.

8.5 Nicotine pharmacokinetics in AGM

Like humans, AGMs eliminate nicotine mainly via metabolism to cotinine in the liver, with CYP2A6 having a major role in mediating this reaction and CYP2B6 having a minor contribution (Schoedel et al., 2003). The $K_m$ for in vitro nicotine metabolism in AGM hepatic microsomes is 29.1 +/- 8.6 μM (Schoedel et al., 2003), which is comparable with the $K_m$ of 64 +/-32.7 μM in human hepatic microsomes (Messina et al., 1997). AGMs and humans have a similar nicotine plasma half-life ranging from 1 to 4 h (Hukkanen et al., 2005; Lee et al., 2006a).
Thesis rationale and overview

Elevated levels of CYP2E1 and CYP2B6 have been detected in the livers and/or brains of heavy alcohol users and smokers, whereas decreased hepatic CYP2A6 activity has been detected in vivo in smokers (Tsutsumi et al., 1989; Benowitz and Jacob, 2000; Hesse et al., 2000; Benowitz et al., 2003; Howard et al., 2003; Miksys et al., 2003). The altered levels of these CYPs which have been detected in heavy alcohol users and smokers could potentially impact the metabolism of certain xenobiotics and affect the efficacy of clinical drugs, risk for drug dependence and susceptibility to toxicity. It is important to understand the factors that contribute to the altered levels of CYPs in alcohol consumers and smokers, as this information can be used to improve our ability to predict who will receive the most therapeutic benefit from certain drugs, whether doses should be adjusted, and who is at increased risk for toxicity from certain compounds.

Studies in rats suggest that ethanol and nicotine may be responsible for the altered levels of CYPs in alcohol users and smokers, respectively. However, there are species differences in the expression, catalytic activity and regulation of CYPs in rodents compared to humans (Martignoni et al., 2006); therefore, experimental results demonstrating the induction/downregulation of CYPs in rats cannot necessarily be extrapolated to humans. Designing a study to directly demonstrate the effects of ethanol and/or nicotine on hepatic and brain levels of CYPs in humans is virtually impossible due to a the presence of confounding factors, as well as technical and ethical limitations. Therefore, we proposed to study the effects of ethanol and/or nicotine on our CYPs of interest in AGM. There are several advantages to using AGM for our investigation. First, AGM are very similar to humans with respect to CYP enzyme expression, substrate specificity and regulation (reviewed in section 8 of introduction), therefore results obtained from our monkey study are relevant to humans. Secondly, using
monkeys, we can conduct a controlled investigation where the exact exposure to nicotine/ethanol is known, confounding factors are minimized, and assessment of protein/mRNA levels, *in vitro* activity and *in vivo* pharmacokinetics can be performed. Such a study would not be possible in humans. Lastly, monkeys will voluntarily consume ethanol at levels comparable to human consumption (Palmour et al., 1997). Therefore, an ethanol self-administration model can be employed.

In my thesis, AGM were used to determine the independent and combined effects of ethanol self-administration and nicotine treatment on:

- Hepatic CYP2E1 levels, and the *in vitro* and *in vivo* metabolism of the CYP2E1 selective substrate chlorozoxazone (**Chapter 1**)
- Hepatic CYP2A6 and CYP2B6 levels, and the *in vitro* and *in vivo* metabolism of nicotine (**Chapter 2**)
- Regional expression of CYP2E1 and CYP2B6 protein in the brain (**Chapter 3**)

**Study Rationales and statement of research hypotheses**

**Chapter 1: The independent and combined effect of ethanol self-administration and nicotine treatment on hepatic CYP2E1 in African green monkeys**

CYP2E1 metabolizes ethanol and several clinical drugs (Table 1) (Lieber, 1997) and the induction of hepatic CYP2E1 could potentially cause metabolic tolerance and altered drug efficacy. CYP2E1 also metabolizes a wide variety of industrial chemicals and environmental pollutants, many of which are metabolically activated by CYP2E1 to generate toxic and carcinogenic metabolites (Table 1) (Lieber, 1997). Furthermore, CYP2E1 mediated metabolism generates ROS, which can cause cellular injury through lipid peroxidation and DNA damage.
Therefore, environmental or genetic factors that increase CYP2E1 activity can potentially increase the risk for chemically mediated toxicity and/or cancer (Trafalis et al.).

Chronic alcohol consumption and cigarette smoking are both known to increase in vivo CYP2E1 activity in humans (Oneta et al., 2002; Benowitz et al., 2003). Ethanol and nicotine can induce hepatic CYP2E1 protein in AGM (Lee et al., 2006c; Ivester et al., 2007), providing strong evidence that these drugs are responsible, at least in part, for the elevated CYP2E1 activity in alcohol consumers and smokers. However, a direct relationship between the ethanol- or nicotine-mediated induction of hepatic CYP2E1 levels and changes in in vivo pharmacokinetics of CYP2E1 substrates has not yet been demonstrated in primates. Furthermore, despite the large proportion of the population that is chronically exposed to both ethanol and nicotine (Falk et al., 2006), no studies have assessed the combined effects of these two drugs on hepatic CYP2E1 levels or in vivo CYP2E1 activity in primates. It is important to characterize the magnitude of hepatic CYP2E1 induction caused by ethanol and nicotine, both alone and in combination, in primates and show that these changes are associated with significant alterations in in vitro metabolism and in vivo disposition to CYP2E1 substrates. This information can be used to model and predict ethanol and/or nicotine mediated changes in the pharmacokinetics of drugs or toxins that could potentially occur in humans. The objective of our study was to determine the independent and combined effects of ethanol self-administration and nicotine treatment on hepatic CYP2E1 protein levels and in vitro activity, as well as in vivo pharmacokinetics of chlorzoxazone (a drug selectively metabolized by CYP2E1).

Hypotheses:
1. Ethanol self-administration and nicotine treatment will independently induce hepatic CYP2E1 protein levels and *in vitro* activity, and will increase the rate of *in vivo* chlorzoxazone metabolism.

2. Combined exposure to ethanol and nicotine will induce hepatic CYP2E1 protein levels and *in vitro* activity, and will increase the rate of *in vivo* chlorzoxazone metabolism; the magnitude of CYP2E1 induction will be greater than with either ethanol or nicotine alone.

**Chapter 2: The differential effects of nicotine treatment and ethanol self-administration on CYP2A6, CYP2B6 and nicotine pharmacokinetics in African green monkeys**

Hepatic CYP2A6 and CYP2B6 can both metabolize a variety of drugs, toxins and endogenous compounds (Table 5 and 9). The downregulation or induction of hepatic CYP2A6 and/or CYP2B6 could potentially affect the metabolism of substrate xenobiotics leading to changes in drug efficacy, altered susceptibility to toxicity and metabolic tolerance. In humans and non-human primates, nicotine is metabolized mainly in the liver by CYP2A6 but to a lesser extent by hepatic CYP2B6 (Yamazaki et al., 1992; Messina et al., 1997; Yamanaka et al., 2005). Since dependent smokers will adjust their smoking behaviour (cigarettes/day, puff volume, puff frequency) to maintain desired nicotine levels (Scherer, 1999), alterations in the activity of hepatic CYP2A6 and/or CYP2B6 could potentially affect nicotine pharmacokinetics and ultimately impact smoking behaviours.

Using tissue from a human liver bank, heavy alcohol consumption was associated with elevated levels of hepatic CYP2A6 and CYP2B6 protein (Hesse et al., 2000; Niemelä et al., 2000). Chronic ethanol administration can induce CYP2A5 in mice and CYP2B1 in rats (Howard et al., 2001; Lu et al., 2011), suggesting that ethanol may be responsible for the elevated levels of CYP2A6 and CYP2B6 in human alcohol consumers. However, due to species differences in the expression and regulation of CYP2A/CYP2B in rodents vs. primates
(Fernandez-Salguero and Gonzalez, 1995b; Maglich et al., 2003; Su and Ding, 2004; Jyrkkärinne et al., 2005), studies should be performed in monkeys to specifically demonstrate the effect of ethanol on hepatic CYP2A6 and CYP2B6 levels and activity as well as nicotine pharmacokinetics in primates. As ethanol is often consumed regularly by smokers, it is important to know if ethanol has the potential to regulate these nicotine metabolizing enzymes.

Cigarette smoking decreases nicotine clearance in humans (Benowitz and Jacob, 2000), which is presumably due to nicotine-mediated downregulation of hepatic CYP2A6, since chronic nicotine treatment decreases CYP2A6 protein levels and *in vitro* nicotine metabolism in monkeys (Schoedel et al., 2003). Smoking does not affect hepatic CYP2B6 protein levels or activity in humans, nor does nicotine treatment affect CYP2B6 protein levels in monkeys (Hesse et al., 2000; Lee et al., 2006b). Although the effect of nicotine on CYP2A6 and CYP2B6 in primates has been established, a relationship between the nicotine-mediated downregulation of hepatic CYP2A6 and changes *in vivo* disposition to CYP2A6 substrates (e.g. nicotine) has not been demonstrated in primates.

Cigarette smoking is more common, heavier and more persistent among individuals with higher alcohol consumption. For example, heavy drinking among smokers has been associated with increased quantity of cigarette use (Batel et al., 1995) and a greater likelihood of remaining a smoker (Myers et al., 2007). It is possible that ethanol-mediated changes in nicotine pharmacokinetics contributes to how alcohol consumption can modify smoking behaviours (Shiffman and Balabanis, 1995). To date, no studies have investigated the effects of combined chronic ethanol and nicotine exposure on CYP2A6, CYP2B6 or nicotine pharmacokinetics in primates.
It is important to characterize the effect of ethanol and nicotine, alone and in combination, on hepatic CYP2A6 and CYP2B6 in primates and determine whether any observed changes in CYP2A6 and/or CYP2B6 expression are associated with significant alterations in in vivo disposition of CYP2A6/CYP2B6 substrates such as nicotine. This information can be used to predict ethanol and/or nicotine mediated changes in the pharmacokinetics of drugs or toxins that are metabolized by CY2A6 and CYP2B6. In this study we used AGM to determine the independent and combined effects ethanol self-administration and nicotine treatment on CYP2A6 and CYP2B6 protein and activity in hepatic liver microsomes. We also assessed the impact of ethanol and nicotine, both alone and combination on the hepatic in vitro metabolism and in vivo pharmacokinetics of nicotine in AGM.

**Hypotheses:**

1. Ethanol self-administration will induce hepatic CYP2A6 and CYP2B6 protein levels and in vitro activity; increase hepatic in vitro nicotine metabolism; and decrease in vivo nicotine AUC.

2. Nicotine treatment will downregulate hepatic CYP2A6, but not CYP2B6, levels and in vitro activity; decrease hepatic in vitro nicotine metabolism; and increase in vivo nicotine AUC.

3. The effects of combined ethanol and nicotine will alter hepatic CYP2A6 and CYP2B6 protein levels and in vitro activity; modify the rate of hepatic in vitro nicotine metabolism; and modify in vivo nicotine disposition.

**Chapter 3: Ethanol self-administration and nicotine treatment induce brain levels of CYP2B6 and CYP2E1 in African green monkeys**

Human alcoholics and smokers have elevated levels of CYP2B6 and CYP2E1 protein in specific regions of the brain (Howard et al., 2003; Miksys et al., 2003). Regional increases in the levels
of CYP2B6 and CYP2E1 in the brain can alter the metabolism of certain centrally-acting xenobiotics, modifying their therapeutic and/or toxic effects. CYP2B and CYP2E1 can be induced by nicotine and/or ethanol in rat brain, suggesting that exposure to these drugs may contribute to the elevated CYP2B6 and CYP2E1 levels observed in tissues from human alcoholics and smokers (Howard et al., 2003; Miksys et al., 2003). However, the regional pattern of CYP2B and CYP2E1 induction by ethanol and/or nicotine in rat brain does not coincide with the specific regions where CYP2B6 and CYP2E1 protein levels are elevated in human alcoholics and smokers (Howard et al., 2003; Miksys et al., 2003), which may be due to species differences (rodent vs primate) in the regulation of brain CYPs.

Monkeys treated with a 0.3 mg/kg bid dose of nicotine showed induction of CYP2B6 and CYP2E1 in several brain regions, the induction was observed in similar but fewer brain regions compared to human smokers (Joshi and Tyndale, 2006a; Lee et al., 2006b). It is possible that the 0.3 mg/kg bid dose of nicotine was too low to produce the regional pattern of CYP2B6 and CYP2E1 induction observed in the brains of humans smokers. Therefore, an objective of the current study was to assess the effect of nicotine at a higher dose of 0.5 mg/kg bid on brain CYP2E1 and CYP2B6 levels in monkeys. We also assessed the effects of ethanol, alone and in combination with nicotine, on brain CYP2B6 and CYP2E1 protein levels in monkeys, which has not been previously studied. This information is important as it will clarify whether ethanol and/or nicotine are responsible for the elevated levels of CYP2B6 and CYP2E1 in the brains of alcohol consumers and smokers. Also, mapping the regional induction of these enzymes and measuring the magnitude of induction will provide some indication of the potential effect these enzymes can have on brain metabolism when induced by ethanol and/or nicotine.
**Hypotheses:**

1. Ethanol self-administration will induce both brain CYP2E1 and CYP2B6 protein in a region and cell-specific manner in monkey brain.

2. Nicotine treatment will induce both brain CYP2E1 and CYP2B6 protein in a region and cell-specific manner in monkey brain.

3. Combined exposure to ethanol and nicotine will induce both CYP2B6 and CYP2E1 in a region- and cell-specific manner in monkey brain; the induction will be greater in magnitude and/or involve more brain region than with either ethanol or nicotine alone.
Section 2. Thesis Chapters

Chapter 1: The independent and combined effects of ethanol self-administration and nicotine treatment on hepatic CYP2E1 in African green monkeys

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Dr. Rachel F. Tyndale, Dr. Sharon Miksys, Dr. Roberta Palmour and Charmaine Ferguson contributed to the experimental design. Dr. Sharon Miksys and Dr. Roberta Palmour conducted the animal study. Charmaine Ferguson conducted all the in vitro experiments, performed the data analysis, and wrote the manuscript. Dr. Bin Zhao performed all HPLC procedures for chlorzoxazone and 6-hydroxychlorzoxazone quantification. Ewa Hoffman assisted with CYP2E1 mRNA quantification. Dr. Rachel F. Tyndale helped with data analysis and editing the manuscript.

Abstract

CYP2E1 metabolizes ethanol and also bioactivates many toxins and procarcinogens. Elevated levels of hepatic CYP2E1 are associated with an increased susceptibility to chemical toxicity and carcinogenesis. This study investigated the induction of hepatic CYP2E1 by ethanol and nicotine, alone and in combination, in a non-human primate model. Monkeys self-administered ethanol and nicotine was administered as subcutaneous injections (0.5 mg/kg bid), alone and in combination compared to control animals (4 groups, N=10/group). Chlorzoxazone was used as a probe drug to phenotype in vivo CYP2E1 activity before and after chronic ethanol and/or nicotine exposure. CYP2E1 protein levels and in vitro chlorzoxazone metabolism were assessed in liver microsomes. Average daily ethanol consumption was \( \approx 3.0 \) g/kg (blood ethanol levels \( \approx 24 \) mmol/L) and was unaffected by nicotine treatment. Ethanol self-administration and nicotine treatment, alone and in combination, significantly reduce plasma chlorzoxazone area under the curve compared to control animals. The effect of ethanol was only observed at higher levels of intake. Ethanol and nicotine increased CYP2E1 protein levels and in vitro chlorzoxazone
metabolism, with combined exposure to both drugs resulting in the greatest increase. The effect of ethanol was also dependent on level of intake. Chronic exposure to ethanol and nicotine induced hepatic CYP2E1 activity and protein levels, particularly when both drugs were used in combination and when ethanol intake was high. These results have important implications for public health, given the association between elevated CYP2E1 and disease, and the large proportion of individuals who are exposed to ethanol and nicotine, often in combination.

Introduction
Cytochrome P450 2E1 is a drug metabolizing enzyme that is responsible for the biotransformation of numerous low-molecular weight compounds, including ethanol, several commonly used industrial solvents, environmental pollutants, and various clinical drugs (Lieber, 1997). Many of these substrates are pro-carcinogens or cytotoxins that are bioactivated by CYP2E1. CYP2E1 is also known to generate high levels of reactive oxygen species that can cause cell damage via lipid peroxidation and DNA strand breaks (Caro and Cederbaum, 2004).

Elevated levels of CYP2E1 are associated with increased susceptibility to chemical toxicity and carcinogenesis. Several polymorphisms have been identified in the human CYP2E1 gene. Individuals with the CYP2E1 RsaI c2 allele, associated with increased CYP2E1 transcriptional activity (Hayashi et al., 1991), are more susceptible to toxicity from industrial chemicals bioactivated by CYP2E1 such as vinyl chloride (Wang et al., 2010c) and n-hexane (Zhang et al., 2006). This and other CYP2E1 genetic variants have been associated with increased risk for hepatocellular (Munaka et al., 2003), colorectal (Morita et al., 2009) and esophageal cancer (Liu et al., 2007).
Hepatic CYP2E1 can be induced by a variety of compounds, many of which are substrates. Ethanol is an inducer of hepatic CYP2E1 protein and activity in humans and monkeys (Lieber, 1997; Ivester et al., 2007). Smoking increases CYP2E1 activity in humans (Benowitz et al., 2003) and chronic nicotine treatment increases hepatic CYP2E1 protein levels and activity in monkeys (Lee et al., 2006c).

Approximately 90% of smokers also consume alcohol (Shiffman and Balabanis, 1995), yet little is known about the combined effects of ethanol and nicotine on hepatic CYP2E1 levels. It was previously shown that rats exposed to both ethanol and nicotine had significantly greater levels of hepatic CYP2E1 protein compared rats exposed to either drug alone (Yue et al., 2009). This enhancement of CYP2E1 protein was partially attributed to a nicotine-stimulated increase in ethanol consumption. There was no indication as to how the observed increases in CYP2E1 protein levels would affect in vitro and in vivo CYP2E1 activity. Understanding the impact of ethanol and nicotine on CYP2E1-mediated metabolism is crucial, given the large proportion of the population that is exposed to both drugs and the potential for elevated CYP2E1 to cause toxicity and disease.

The AGM is established as an excellent animal model of human CYP2E1 expression and activity (Lee et al., 2006c). Chlorzoxazone (CZN), a clinically used muscle relaxant that is metabolized by CYP2E1 to 6-hydroxychlorzoxazone (6OHCZN), is a validated probe drug for the measurement of CYP2E1 activity in both humans and monkeys (Ernstgard et al., 2004; Lee et al., 2006c). As in other species, CZN-6-hydroxylation in monkey liver microsomes can be inhibited by anti-CYP2E1 antibodies and selective chemical inhibitors of CYP2E1 providing evidence that CYP2E1 is the primary enzyme involve in the 6-hydroxylation of CZN (Amato et al., 1998). AGMs are also useful in modeling human alcohol consumption. These monkeys will
voluntarily self-administer ethanol at levels comparable to human consumption and are therefore routinely used in alcohol research (Palmour et al., 1997).

We investigated the independent and combined effects of ethanol self-administration and nicotine treatment on *in vivo* CZN disposition, hepatic CYP2E1 protein levels and *in vitro* CZN metabolism. We hypothesized that ethanol and nicotine would independently induce CYP2E1 levels resulting in increased CZN clearance *in vivo* and *in vitro* CZN metabolism. In addition, combined ethanol and nicotine exposure was hypothesized to result in greater induction of CYP2E1 protein and *in vitro* CZN activity compared to either drug alone, due to both the direct effects of the inducers and a nicotine-mediated increase in ethanol consumption. The induction of CYP2E1 by ethanol is dose-dependent in humans (Millonig et al., 2011), therefore we also investigated if the effects of ethanol were dependent on the level of ethanol intake.

**Methods**

**Materials.** CZN and 2-benzoxazolinone were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All other chemicals were obtained from standard commercial sources. Protein estimation was performed with dye reagent purchased from Bio-Rad Laboratories (Hercules, CA). Prestained molecular weight protein markers were purchased from MBI Fermentas (Flamborough, ON, Canada). Hybond nitrocellulose membrane was purchased from Amersham Biosciences (Toronto, ON, Canada). Human cDNA expressed CYP2E1, CYP2A6, CYP1A1, CYP1A2, CYP2D6, CYP3A4 and CYP2B6 were purchased from BD Biosciences (Franklin Lakes, NJ). Polyclonal anti-rat CYP2E1 antibody was purchased from Fitzgerald Industries (Acton, MA). Horseradish peroxidase-conjugated anti-sheep secondary antibody was purchased from Millipore. (Billerica, MA). Chemiluminescent substrate was purchased from
Pierce Chemical Company (Rockford, IL). Autoradiographic film was purchased from Ultident (St. Laurent, PQ, Canada).

**Animals.** Adult male African green monkeys ( vervets, *C. aethiops*) were housed outdoors in social groups at Caribbean Primates Ltd (St Kitts). They were acquired from a large, isolated, and non-endangered Caribbean population (Palmour et al., 1997). Monkeys were given standard rations of Purina monkey chow supplemented with fresh fruit and vegetables twice a day and were allowed to feed ad libitum. Drinking water was also available ad libitum.

**Drug Treatment.** The study timeline is shown in Figure 9. The first 14 days of the study consisted of an ethanol preference screening phase, where monkeys were given access to 10% v/v ethanol in 0.5% w/v sucrose solution for 4 hours per day. Forty monkeys that voluntarily consumed more than 1g ethanol/kg/day were selected and randomized into four groups based on daily ethanol consumption (n=10 per group); a control (no drug), ethanol-only, nicotine-only and ethanol + nicotine groups. The following 14 days (day 15-28) consisted of a washout period, where monkeys had no exposure to ethanol or nicotine. During the second phase of the study, from day 29-42, monkeys in the ethanol-only and ethanol + nicotine groups were allowed to self-administer 10% ethanol in 0.5% sucrose solution for 4 hours a day while the other groups consumed 0.5% sucrose solution on the same schedule. During the third phase of the study, from day 43-63, in addition to ethanol (or sucrose), monkeys in the nicotine-only and ethanol + nicotine were given s.c injections of nicotine bitartrate (milligram base in saline, pH 7.0) twice daily at a dose of 0.05 mg/kg on day 43, 0.1 mg/kg on day 44, 0.25 mg/kg on day 45, and 0.5 mg/kg for subsequent days. The first injection was given 30 min prior to the ethanol (or sucrose)
access period. The second injection was given 10 hours later. Monkeys in ethanol-only and control groups were given saline injections (as a vehicle control for nicotine bitartrate) on the same schedule. On day 50, nicotine treatment and ethanol access was suspended in order to conduct pharmacokinetic testing. Monkeys received 7 mg/kg CZN intragastrically under ketamine anesthesia, and blood samples were drawn at t-10 min (10 minutes before CZN administration), 10, 20, 30, 60, 120, 240, and 360 min after CZN administration. Blood samples (2 ml) were drawn immediately after the ethanol access period on days 38 and 59 to determine blood ethanol levels. All blood samples were centrifuged and the plasma removed and frozen for subsequent drug analyses. A commercial enzymatic assay kit (Sigma Diagnostics, St Louis, MO) was used to determine plasma blood levels of ethanol. Body weights at the start of the study (average = 5.9 kg ± 0.5) were not significantly different from body weights at sacrifice (average = 5.7 kg ± 0.5) (paired t-test, p>0.5). There were no significant differences in body weights between groups at the start of the study (one way analysis of variation (ANOVA), F (3, 36) = 0.3784, p>0.05) or at sacrifice (one way ANOVA, F (3, 36) = 1.860, p>0.05). Animals were sacrificed by exsanguination via the femoral artery under ketamine anesthesia and livers were immediately dissected and flash-frozen in liquid nitrogen and stored at -80°C until further use. Upon visual inspection of the livers, there were no apparent signs of injury or cirrhosis. The experimental protocol was reviewed and approved by the Institutional Review Board of Behavioural Sciences Foundation and the University of Toronto Animal Care Committee. All the procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care.
Monkeys were randomized into four study groups (n=10 per group); Study groups consisted of a no-drug control group, an ethanol-only group, a nicotine-only group and an ethanol + nicotine group. Groups were named based on drug exposure during the last phase of the study (phase III). Blood ethanol levels (BEL) were measured near the end of phase II and phase III. In vivo CZN metabolism was assessed during the washout period prior to phase II, and at the end of phase III. On day 50, nicotine treatment and ethanol access was suspended in order to conduct pharmacokinetic testing.

Figure 9. Overview of study timeline.
**In Vivo CZN Plasma Assessments.** Plasma CZN levels were assayed based on the methods in Howard et al. (2001). Briefly, plasma was centrifuged at 3000g for 10 min, after which 100 µl of the supernatant was removed and incubated with β-glucuronidase (10 mg/ml, 50 µl) overnight at 37°C. Following incubation, internal standard of 2-benzoazolinone (2.0 mM, 25 µl), 10% perchloric acid (120 µl) and ethyl acetate/hexane (3 ml) were added, the sample was shaken for 30 min, centrifuged at 3500g for 15 min, and the organic phase was evaporated to dryness at 37°C. The sample was reconstituted into 110 µl of mobile phase consisting of 50 mM ammonium acetate (adjusted to pH to 4.0 with 1 M glacial acetic acid)/acetonitrile (65:35). CZN was measured by high-performance liquid chromatography with UV detection at 287 nm. An Agilent Zorbax SB-C18 column (5 µm, 4.6 x 250 mm) (Agilent Technologies, Palo Alto, CA) was used to separate CZN and 2-benzoazolinone using a flow rate of 0.7 ml/min. The retention times for CZN and 2-benzoazolinone were 18.5 and 10.1 min, respectively.

**Microsomal Membrane Preparation.** Monkey liver tissue was homogenized in 100 mM Tris, 0.1 mM ethylenediaminetetraacetic acid, 0.1 mM dithiothreitol, and 0.32 M sucrose (pH 7.4) for immunoblotting, or in 1.15% w/v KCl for *in vitro* metabolism assessments and then centrifuged at 12,500g for 30 min at 4°C. The supernatant was then centrifuged at 110,000g for 90 min at 4°C, and the pellet was resuspended in 100 mM Tris, 0.1 mM ethylenediaminetetraacetic acid, 0.1 mM dithiothreitol, 1.15% w/v KCl, and 20% v/v glycerol for immunoblotting or 1.15% w/v KCl for *in vitro* metabolism assays. The protein content of liver microsomes was assayed with the Bradford technique using a Bio-Rad Protein Assay kit. Microsomes were stored at −80°C.
**In Vitro CZN and 6OHCZN Assessments.** CZN 6-hydroxylation was assayed according to the protocol established by Leclercq et al., in which the protein concentration and incubation times were optimized for linear formation of 6OHCZN (Leclercq et al., 1998). Monkey hepatic microsomal protein (0.4 mg) was mixed with 0.1 M Tris buffer at pH 7.6, 10 mM magnesium chloride, 5 mM NADPH and 950 µM CZN to a final volume of 500 µl. The reaction mixture was incubated for 20 min at 37°C. Zinc sulfate (15% w/v, 0.2 ml) was added to stop the reaction, and 6.4 µg of the internal standard, 2-benzoxazolinone in Tris buffer, was added per reaction. Following centrifugation for 10 min at 12,700g, the supernatant was injected onto an Agilent Zorbax SB-C18 column (5 µm, 4.6 x 250 mm) (Agilent Technologies, Palo Alto, CA) with UV detection at 287 nm. The mobile phase consisted of 50 mM ammonium acetate (adjusted to pH to 4.0 with 1 M glacial acetic acid)/acetonitrile (65:35) with a flow rate of 0.7 ml/min. The retention times for CZN, 6OHCZN, and 2-benzoxazolinone were 18.5, 7.5, and 10.1 min, respectively. The absolute and relative recoveries of 6OHCZN were 90.4% and 99.4% respectively.

**Immunoblotting.** Monkey liver microsomal protein was serially diluted to generate a standard curve and to establish the linear detection range for the immunoblotting assays. To determine cross-reactivity of the primary antibodies, cDNA-expressed human CYP2E1, CYP2A6, CYP1A1, CYP2A2, CYP2D6, CYP3A4 and CYP2B6 were used as positive or negative controls. Liver microsomal proteins (4 ug) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 8% separating gels), and then transferred overnight onto nitrocellulose membranes. Gels were stained with Coomassie blue R-250 to ensure equal loading of protein among lanes. To detect hepatic CYP2E1, the membranes were first blocked
with 1% skim milk in 50 mM Tris-buffered saline (TBS) containing 0.1% w/v bovine serum albumin, 0.1% v/v Triton X-100 for 1 hr. Membranes were then incubated with anti-CYP2E1 antibody diluted 1:1000 for 2 h, followed by three 5 min washes with TBS containing 0.1% v/v Triton X-100. The membranes were then blocked again with the initial blocking solution for 1 hr and then incubated with peroxidase-conjugated rabbit anti-sheep antibody diluted 1:5000 for 1 hr, followed by three 5 min washes with TBS containing 0.1% v/v Triton X-100. Proteins were visualized using chemiluminescence followed by exposure to autoradiography film. Immunoblots were analyzed using MCID Elite software (InterFocus Imaging Ltd., Linton, UK), and the relative density of each band was expressed as arbitrary density units after subtracting background.

**Isolation, cDNA synthesis and mRNA quantification.** Liver tissue (50-100 mg) was homogenized in TRIzol reagent (Invitrogen) and total RNA was isolated according to the TRIzol reagent protocol. RNA concentrations were determined spectrophotometrically and total RNA integrity was confirmed by electrophoresis on a 1.2% agarose gel (OnBio, ON, Canada) stained with ethidium bromide and inspection of the 28S and 18S ribosomal bands. cDNA was synthesized using 1 μg of total RNA, random hexamers (Invitrogen), RiboLock RNase inhibitor (Fermentas, Burlington, ON) and MMLV Reverse Transcriptase (Invitrogen) according to protocols provided by the manufacturers. Primers for real-time PCR amplification of CYP2E1 and β-actin were as follows: CYP2E1 forward primer (CYP2E1ex1): 5’-CCG CTT CCC ATC GGG AAC-3’, CYP2E1 reverse primer (CYP2E1ex1): 5’-GCG CTT TCA CCG CCT TGT A-3’, β-actin forward primer (ACTBFex3): 5’-CAG AGC AAG AGA GGC ATC CT- 3’, β-actin reverse primer (ACTBRex4): 5’-GGT CTC AAA CAT GAT CTG GGT C-3’. The
sequence of CYP2E1 in African green monkey is not known; primer specificity was based on human CYP2E1 and Rhesus Macaque (*Macaca mulatta*) CYP2E1. Amplification and fluorescence detection were performed using the ABI ViiA7 Real-Time PCR system (Life Technologies). Real-time PCR amplification mixture (20 μl) contained 1 μl of synthesized cDNA, 10 μl of 2× Fast SYBR-Green Mix (Life Technologies), and 0.3 μM of each primer. Cycling conditions consisted of an initial activation of AmpliTaq Fast DNA polymerase followed by 40 cycles of denaturing (95°C for 15 s) and annealing/extension (58°C for 20s). Dilutions of monkey and human cDNA were used to determine the range of log linear detection. CYP2E1 mRNA levels were obtained by normalized to β-actin and using the comparative CT method for relative quantification as described by the manufacturer (Real-Time PCR Chemistry Guide, Life Technologies)

**Statistics.** Differences in ethanol (or sucrose) consumption and blood ethanol levels were assessed by one-tailed Student’s t-test, unpaired tests for between group comparisons and paired tests for within group comparisons. All pharmacokinetic statistical analyses were performed using SAS software version 8.2 (SAS Institute, Cary, NC). One-tailed paired Student’s t-tests were used to assess differences in *in vivo* CZN pharmacokinetic parameters measured pre and post-drug administration. One tailed unpaired Student’s t-test was used to compare the change in CZN AUC\(_{0-6hr}\) among groups. One-way ANOVA followed by post-hoc tests (Kruskal Wallis and test for linear trend) were used to determine differences in CYP2E1 protein levels, mRNA levels and *in vitro* CZN metabolism between groups. Correlations were calculated with Pearson correlation coefficients.
Results

Monkeys Voluntarily Self-Administered Ethanol.

Monkeys consistently self-administered high levels of 10% ethanol throughout the study (Figure 10A). Mean daily consumption of 10% ethanol in sucrose solution during the four hour sessions in phases II and III ranged from 23.6-54.6 ml/kg (1.9-4.4g ethanol/kg), resulting in an average consumption of 38.12 ± 7.8 ml/kg (3.0g of ethanol/kg). A gradual increase in mean daily ethanol consumption was observed as monkeys progressed from phase II to phase III of the study. This increase was statistically significant for both the ethanol-only (p<0.001) and the ethanol + nicotine groups (p=0.04) (Table 12). There was no significant difference in mean ethanol consumption between the ethanol-only and the ethanol + nicotine groups (Phase III, Table 12), indicating no effect of nicotine treatment on voluntary ethanol consumption. Sucrose consumption remained constant throughout the study.
Figure 10. Monkeys voluntarily self-administered ethanol.
A) Average daily consumption by monkeys self-administering ethanol in sucrose (squares) or sucrose alone (circles). Phase I is the ethanol preference screening period (consumption levels at screening represent average consumption during this phase). During phase II monkeys in the ethanol-only and ethanol + nicotine groups self-administered ethanol in sucrose solution while the other groups consumed sucrose solution only on the same schedule. During Phase III in addition to ethanol (or sucrose), monkeys in the in the nicotine-only and ethanol + nicotine groups were given nicotine injections while monkeys in the other groups were given saline injections on the same schedule. B) A median split was used to categorize monkeys into low and high ethanol consumers based on mean daily consumption of 10% ethanol during phase II and III. B) Unfilled symbols represent monkeys in ethanol-only group, filled symbol represent monkeys in the ethanol + nicotine group and horizontal lines indicated group means. Significantly different from low consumers, *p<0.05.
Table 12. Consumption of 10% ethanol increased over time with no increase in blood ethanol levels (values expressed as mean ± SE, n=10/group)

<table>
<thead>
<tr>
<th></th>
<th>Phase II</th>
<th></th>
<th>Phase III</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Consumption (ml/kg/day)</td>
<td>BEL (mmol/L)</td>
<td>Consumption (ml/kg/day)</td>
<td>BEL (mmol/L)</td>
</tr>
<tr>
<td>EtOH-only</td>
<td>35.4 ± 1.2</td>
<td>24.6 ± 3.9</td>
<td>42.4 ± 1.9*</td>
<td>26.3 ± 9.3</td>
</tr>
<tr>
<td>EtOH + Nicotine</td>
<td>37.8 ± 2.2</td>
<td>23.8 ± 2.1</td>
<td>40.7 ± 3.1*</td>
<td>29.7 ± 4.7</td>
</tr>
<tr>
<td>Low consumers</td>
<td>32.9 ± 1.6</td>
<td>22.9 ± 3.8</td>
<td>35.5 ± 1.6*</td>
<td>24.7 ± 4.0</td>
</tr>
<tr>
<td>High consumers</td>
<td>37.3 ± 1.6†</td>
<td>25.5 ± 2.2</td>
<td>45.1 ± 1.9* †</td>
<td>31.7 ± 4.0</td>
</tr>
</tbody>
</table>

BEL: blood ethanol level
Consumption and BEL were not significantly different between groups during either phase
* p<0.05, † p<0.05, ‡ p<0.001, significantly different from low consumer
Considerable individual variation in ethanol intake was observed. Monkeys were divided into high and low ethanol consumers by performing a median split based on mean daily consumption of ethanol during phase II and III (Figure 10B and Table 12).

Blood ethanol levels were measured on two occasions, once during phase II (day 38) and again during phase III (day 59). A paired-test reveals no significant difference in blood ethanol levels measured during phase II and phase III for either the ethanol-only and ethanol + nicotine groups or for the high and low consumer groups, despite increases in ethanol consumption (Table 12).

**In Vivo CZN Disposition is Influenced by Nicotine Treatment and the Level of Daily Ethanol Intake**

A within animals design was used to assess changes in *in vivo* CZN metabolism due to animal variation in CZN pharmacokinetics. A comparison of *in vivo* CZN metabolism pre- and post-drug administration indicated that the nicotine-only group had a 34% decrease in CZN area under the curve (AUC) (p=0.002) and a 42% reduction in the maximum plasma CZN concentration (C\text{max}) (p<0.001) compared to before treatment (Table 13). Mean values for all the assessed CZN pharmacokinetic parameters were not significantly altered in the ethanol-only group; however, several monkeys showed a substantially decreased CZN AUC after ethanol self-administration (Figure 11). In the ethanol + nicotine group there was a significant reduction in CZN AUC by 27% (p=0.02), C\text{max} by 35% (p=0.003) and the time to maximum CZN concentration (T\text{max}) by 35% (p=0.04). Kinetic parameters were not significantly different between groups pre-drug administration (day 22) and control monkeys did not show significant changes in CZN pharmacokinetic parameters assessed on day 22 compared to day 50.
Table 13. Kinetic parameters (mean ± SE, n=10) from CZN pre-drug administration (day 22) and post-drug administration (day 50)

<table>
<thead>
<tr>
<th></th>
<th>Pre-EtOH</th>
<th>Post-EtOH</th>
<th>Pre-nicotine</th>
<th>Post-nicotine</th>
<th>Pre-EtOH + nicotine</th>
<th>Post-EtOH + nicotine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AUC (hr.ug/ml)</strong></td>
<td>54.8 ± 7.9</td>
<td>56.6 ± 13.6</td>
<td>44.8 ± 6.2</td>
<td>29.5 ± 5.6*</td>
<td>56.8 ± 4.6</td>
<td>41.6 ± 3.2*</td>
</tr>
<tr>
<td><strong>Cmax (ug/ml)</strong></td>
<td>17.5 ± 2.7</td>
<td>27.7 ± 5.7</td>
<td>20.2 ± 3.6</td>
<td>11.7 ± 2.5*</td>
<td>25.3 ± 3.0</td>
<td>16.4 ± 2.0*</td>
</tr>
<tr>
<td><strong>tmax (hr)</strong></td>
<td>1.3 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>0.7 ± 0.1*</td>
</tr>
<tr>
<td><strong>t1/2 (hr)</strong></td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.8 ± 0.3</td>
</tr>
</tbody>
</table>

Kinetic parameters were not significantly different between groups pre-drug administration

*p<0.05, significantly different from pre-drug administration within the same treatment group
Figure 11. Ethanol self-administration and nicotine treatment reduced CZN AUC.
Ethanol self-administration and nicotine treatment reduced chlorzoxazone AUC. A) Mean CZN plasma concentration over time curves for treatment groups, n=10 per group. B) Lines represent the change in plasma CZN AUC$_{0-6h}$ for individual monkeys pre-drug administration (day 22) and post-drug administration (day 50). Diamonds with error bars represent mean AUC$_{0-6h}$ ± SE for the group pre- and post drug administration; for the ethanol-only group, the filled diamonds indicate the mean including the monkey with a noticeably high CZN AUC$_{0-6h}$ both pre- and post-ethanol exposure (indicated by the dashed line) and the unfilled diamonds indicate the mean excluding this monkey. Significantly different from same group of monkeys pre-drug administration, *p<0.05.
There was a significant correlation between post-ethanol CZN AUC\textsubscript{0-6h} and mean daily consumption of 10 % ethanol (r = 0.42, p = 0.03) (Figure 12A). The high ethanol consumers had a post-ethanol CZN AUC\textsubscript{0-6h} of 34 h.µg/ml, which was significantly lower than the CZN AUC\textsubscript{0-6h} of 64 h.µg/ml seen in the low consumers (p<0.05) (Figure 12B). Only in the high consumer group was there a significant change in CZN AUC post ethanol consumption (p<0.05) (Figure 12C). Taken together, these results show a reduction in CZN AUC by ethanol that is dependent on the level of intake.
**Figure 12. Correlation between ethanol consumption and CZN AUC.**

A) There was a significant negative correlation between mean consumption of 10% ethanol (ml/kg/day) during phase II and II and CZN AUC\textsubscript{0-6hr} post-drug administration (day 50). With the exclusion of the monkey with noticeably high post-ethanol CZN AUC (circled), the correlation remains significant (r=0.43, p=0.04). B) High ethanol consumers (see Figure 10B) had a significantly reduced CZN AUC\textsubscript{0-6hr} compared low consumers (p<0.05), n=10 per group. C) Lines represent the change in plasma CZN AUC\textsubscript{0-6hr} for high or low ethanol consumers pre-drug administration (day 22) and post-drug administration (day 50). Diamonds with error bars represent mean AUC\textsubscript{0-6hr} ± SE for the group pre- and post drug administration with (filled) or without (unfilled) the inclusion of a monkey with a noticeably high CZN AUC\textsubscript{0-6hr} both pre- and post-ethanol exposure (indicated by the dashed line). Significantly different from same group of monkeys pre-drug administration, *p<0.05.
Hepatic CYP2E1 Protein Levels are Induced by Ethanol and Nicotine, Particularly when Both Drugs are Present in Combination and at Higher Ethanol Intakes

An immunoblotting assay was established to measure CYP2E1 protein levels in monkey liver. Detection of CYP2E1 in serially diluted liver microsomal protein from a control monkey was shown to be linear from 2-20 µg of protein (Figure 13A). All subsequent immunoblots were loaded with 5 µg of microsomal protein. The CYP2E1 antibody did not cross-react with other cDNA-expressed human cytochromes P450 and monkey hepatic CYP2E1 co-migrated with cDNA-expressed human CYP2E1 (Figure 13B).
Figure 13. Detection of CYP2E1 monkey hepatic protein was linear and specific.
A) A dilution curve of monkey hepatic microsomal protein showed linear detection of CYP2E1 from 2-20 µg. B) The anti-CYP2E1 antibody used for the CYP2E1 immunoblotting did not cross react with cDNA-expressed human CYP2A6, CYP2A1, CYP1A2, CYP2D6, CYP3A4, CYP2B6. Monkey hepatic CYP2E1 co-migrated with cDNA-expressed human CYP2E1.
Compared to control monkeys, ethanol self-administration alone resulted in a 56% increase in CYP2E1 levels (p<0.05), nicotine treatment alone resulted in a 55% increase (p<0.05), and combined ethanol self-administration and nicotine treatment resulted in a 106% increase (p<0.001), suggesting an additive effect (Figure 14A and 14B). Comparing mean CYP2E1 levels across all the groups, there is a significant linear trend; ethanol or nicotine exposure alone increased CYP2E1 levels compared to no drug exposure, and CYP2E1 levels were further increased with combined exposure to both drugs (p_trend=0.0002). Ethanol and nicotine exposure, either alone or in combination, did not significantly alter CYP2E1 mRNA levels in the liver (F(3, 33)=2.423, p=0.083).

The effect of ethanol consumption level on CYP2E1 protein levels was also examined. High ethanol consumers had a 97% increase in CYP2E1 levels relative to control monkeys, while low consumers had a 65% increase. Comparing mean CYP2E1 levels among monkeys in the control, low ethanol consumer and high ethanol consumer groups, there was a significant linear trend, indicating increasing CYP2E1 protein levels with higher levels of ethanol consumption (p_trend = 0.0004) (Figure 14C). CYP2E1 mRNA levels in the liver were not significantly different among the high consumer, low consumers or control animals (F(2, 27)=2.349, p=0.1147).
Figure 14. Ethanol self-administration and nicotine treatment, alone and in combination, induced hepatic CYP2E1 protein levels.
A) Representative immunoblot for hepatic CYP2E1 protein from monkeys in the four study groups (showing n = 4 of 10 per group). Coomassie blue staining was used to confirm equal protein loading among lanes. B) Mean percent increase in CYP2E1 protein relative to control group. A significant linear trend was observed, with animals in the ethanol + nicotine group having the highest levels of CYP2E1. Significantly different from control group, *p<0.05, **p<0.001. n=10 per group. C) Mean percent increase in hepatic CYP2E1 protein in high and low ethanol consumers (see Figure 10B) relative to control group. A significant linear trend was observed, demonstrating increased CYP2E1 levels with higher levels of ethanol consumption. Significantly different from control group,*p<0.05, **p<0.001. n=10 for each group.
In Vitro CZN Metabolism is Induced by Ethanol and Nicotine, Particularly when Both
Drugs are Present in Combination and at Higher Ethanol Intakes

The rate of CZN metabolism to 6OHCZN was measured in vitro using monkey liver
microsomes. There was a significant positive correlation between hepatic CYP2E1 levels and in
vitro 6OHCZN formation velocity at 950 µM (approximate Vmax) (r=0.45 p=0.002) (Figure
15A). Compared to control monkeys, ethanol self-administration alone and nicotine treatment
alone resulted in an 11% increase in 6OHCZN formation velocity, although these increases were
not significant after post-hoc testing (p>0.05) (Figure 15B). Combined ethanol self-administration
and nicotine treatment resulted in a 21% increase (p<0.05), suggesting an additive
effect. Comparing mean CZN metabolism velocity across all the groups, there is a significant
linear trend, with monkeys in the ethanol and nicotine combined group having the highest rate of
CZN metabolism (p_trend=0.003).

The impact of ethanol consumption level on in vitro CZN metabolism was investigated.
High ethanol consumers had a 19% increased in velocity of CZN metabolism compared to
control monkeys, whereas the low consumers had a 13% increase. Comparing mean CZN
metabolism velocity among monkeys in the control, low ethanol consumer and high ethanol
consumer groups, there was a significant linear trend, demonstrating that the rate of CZN
metabolism increases with higher levels of ethanol consumption (p_trend=0.003) (Figure 15C).
Figure 15. Ethanol self-administration and nicotine treatment increased *in vitro* CZN metabolism.

A) Hepatic CYP2E1 protein levels were positively correlated with 6OHCZN formation velocity at 950 µM CZN (approximate vmax). B) Mean percent increases in the 6OHCZN formation velocity relative to the control group. A significant linear trend was observed, with animals in the ethanol + nicotine group (group 4) having the highest velocity. Significantly different from control group, *p<0.05. n=10 per group. C) Mean percent increases in velocity of CZN metabolism in high and low ethanol consumers (see Figure 10B.) relative to the control group. A significant linear trend was observed, demonstrating increased CZN velocity with higher levels of ethanol consumption. Significantly different from control group,*p<0.05. n=10 for each group.
Discussion

Humans and AGM have similar hepatic CYP2E1 levels, *in vivo* CZN disposition and *in vitro* CZN metabolism (Hayashi et al., 1991; Lee et al., 2006c), and while the AGM CYP2E1 has not been sequenced, both the cynomolgus monkey (Macaca fascicularis) and the rhesus monkey (Macaca mulatta), which are closely related to AGM, have a greater than 90% amino acid homology to human CYP2E1. Thus, AGM offer a valuable model for predicting the impact of ethanol and nicotine on CYP2E1 induction and metabolism in humans.

Monkeys consistently self-administered high levels of ethanol throughout the study. Previous alcohol research classified AGM voluntarily self-administering between 0.8-3.5 g/kg ethanol/day as moderate consumers (Palmour et al., 1997). In our study, average daily ethanol intake was approximately 3.0 g/kg, identifying these monkeys as moderate consumers.

Moderate alcohol consumption in humans has been described as intakes resulting in blood ethanol levels ranging from 5-20 mmol/L (Eckardt et al., 1998). In our study, monkeys achieved an average blood ethanol level of 26 ± 2.55 mmol/L comparable to moderate consumption in humans; using blood ethanol level to compare ethanol consumption takes into consideration the two-fold greater rate of ethanol elimination in AGM compared to humans (Ervin et al., 1990).

Ethanol consumption steadily increased throughout the study, whereas blood ethanol levels remained unchanged over time, suggesting increased rates of ethanol elimination in the monkeys. In humans, rats and other mammals ethanol metabolism is primarily mediated by alcohol dehydrogenase and to a lesser extent by CYP2E1 (Matsumoto et al., 1996; Lands, 1998). Human alcoholics have higher levels of CYP2E1, no change in alcohol dehydrogenase and an increased capacity to eliminate ethanol compared to non-alcoholics, suggesting that elevated CYP2E1 levels can affect ethanol metabolism (Vidal et al., 1990). Based on this premise,
induction of CYP2E1 in the ethanol-consuming monkeys may have contributed to metabolic tolerance, allowing monkeys to consume more ethanol without a corresponding rise in blood ethanol level.

Nicotine treatment did not affect voluntary consumption of 10% ethanol in AGM. Some studies in rats have shown an increase in ethanol consumption with chronic or repeated nicotine treatment while others have shown that nicotine treatment has no effect or even decreases ethanol consumption (Blomqvist et al., 1996; Le et al., 2000; Olausson et al., 2001; Sharpe and Samson, 2002). Differences in rat strains, methods used to initiate ethanol consumption and duration of the ethanol access period may contribute to the inconsistent results. A limited number of human studies have investigated the effect of nicotine on alcohol consumption. Occasional smokers (smoking an average of 10 cigarettes per week, and only smoking on two days per week) consumed more alcohol when smoking nicotine-containing cigarettes compared to de-nicotinized cigarettes (Barrett et al., 2006). A similar study in regular smokers (smoking more than 10 cigarettes per day) showed that smokers deprived of nicotine had a greater urge to consumed alcohol compared to non-deprived smokers (Cooney et al., 2003). Therefore, the effect of nicotine on ethanol consumption in either rats or humans is complex and requires further investigation. Our study is the first to look at the effect of nicotine on voluntary ethanol self administration in monkeys where we observed no significant increase or decrease in ethanol consumption from the nicotine administration under the conditions tested.

Here we show that variation in ethanol intake, within the range of moderate drinking, can lead to very different CYP2E1 levels and activity as measured by CZN metabolism. High ethanol consuming monkeys were only drinking 25% more ethanol per day compared to the low consumers; however, they demonstrated nearly a 50% greater reduction in post-ethanol CZN
AUC in vivo. These results are interesting from a public health perspective, as our results suggest that among moderate consumers those with higher daily ethanol intakes may be at elevated risk for CYP2E1 associated diseases.

The average smoker has a total daily nicotine intake of 0.2 to 1.1 mg/kg (Benowitz and Jacob, 1984) resulting in plasma levels of 10-50 ng/ml during the day (Benowitz, 1990). The total daily nicotine dose administered to the monkeys was at the high end of this range (1.0 mg/kg/day) to compensate for the slightly faster nicotine metabolism in AGM (Lee et al., 2006b). Although the pattern of nicotine intake in this study differs from smoking, the levels and duration of nicotine in the plasma were estimated to be comparable to what is observed in human smokers (Benowitz, 1990; Lee et al., 2006b). Nicotine increased CYP2E1 protein, CYP2E1 in vitro activity, and in vivo CZN clearance in the monkeys, consistent with the results of a previous study that assessed the effect of chronic nicotine treatment on CYP2E1 and CZN disposition in African green monkeys (Lee et al., 2006c). The increase in CZN clearance following chronic nicotine exposure, without any change in CZN half-life, suggests that nicotine increases CZN first pass metabolism. It is unlikely that the change in CZN clearance could be attributed to nicotine’s effect on blood flow. Monkeys were administered CZN more than 12 hr after the last nicotine injection, at which point any effect on hepatic blood flow would have subsided (Hashimoto et al., 2004). In humans, smoking increases CZN clearance by 25% (Benowitz et al., 2003). Nicotine-treated monkeys, similarly, showed a 34% decrease in CZN AUC, supporting a role for nicotine as the CYP2E1-inducing agent in cigarette smoke. Our results are not consistent with the conclusions from a recent study showing that administration of a 42-mg transdermal nicotine patch twice a day for 10 days did not affect CZN clearance in humans (Hukkanen et al., 2009). This discrepancy may be due to differences in the dose and duration of
nicotine achieved in the plasma with a transdermal nicotine patch versus smoking, which our study was designed to model.

There was a trend toward monkeys in the ethanol and nicotine combined group having the highest CYP2E1 protein levels and fastest rates of in vitro CZN metabolism compared to monkeys in either the ethanol-only or nicotine-only groups. This trend was not present in the in vivo CZN pharmacokinetic parameters, which may be due to the timing of the CZN pharmacokinetic testing. The post-drug CZN challenge was performed on day 50 and protein levels and in vitro activity were assessed in tissue from monkeys sacrificed on day 64. Ongoing induction of CYP2E1 by ethanol and nicotine may have occurred between days 50 and 64 of the study, resulting in a lack of correlation between in vivo and ex vivo assessment of CYP2E1 activity. In humans, moderate consumption of alcohol (40 g/day) over a period of four weeks resulted in a gradual increase in CYP2E1 activity measured by CZN clearance. CZN clearance was significantly faster on day 28 compared to day 21 (Oneta et al., 2002). In rats, 7 days of nicotine treatment is sufficient to induce CYP2E1 levels in the liver; however, whether there is further induction of CYP2E1 beyond 7 days is not known (Micu et al., 2003). Previously a correlation between in vivo CZN clearance and CYP2E1 protein levels was shown in nicotine-treated monkeys; however, the duration between the in vivo and ex vivo testing was substantially shorter compared in this study (7 vs. 14 days) (Lee et al., 2006c).

Hepatic CYP2E1 mRNA levels were not significantly altered by nicotine or ethanol exposure, suggesting that, at these doses and durations of ethanol and nicotine exposure, induction of CYP2E1 protein occurs via a non-transcriptional mechanism. Consistent with our findings, ethanol-treated rats with blood ethanol levels similar to our monkeys showed induction of hepatic CYP2E1 without a corresponding increase in CYP2E1 mRNA levels (Ronis et al.,
Ethanol induces CYP2E1 protein levels in rats by protecting the enzyme from degradation (Roberts et al., 1995). Nicotine does not induce rat hepatic CYP2E1 via transcription or by protein stabilization and may involve an increase in translational efficiency (Wu et al., 1997) (Micu et al., 2003). Thus ethanol and nicotine may increase CYP2E1 in monkey liver using similar post transcriptional mechanisms as observed for rat liver CYP2E1.

In conclusion, ethanol and nicotine increased hepatic CYP2E1 protein and in vitro CZN metabolism leading to increased CZN clearance in vivo. The effect of ethanol was dependent on the level of daily ethanol intake and combined exposure to ethanol and nicotine resulted in the highest levels of hepatic CYP2E1 protein and activity. Nicotine treatment did not affect ethanol consumption. The induction of hepatic CYP2E1 by nicotine and ethanol may mediate some of the negative health effects of smoking and alcohol consumption via increased metabolic bioactivation of many commonly used industrial chemicals, environmental pollutants and drugs into toxic metabolites (Lieber, 1997). Since a large proportion of the population is exposed to ethanol, nicotine or often both, these findings have important implications for public health, health risk assessment and disease prevention.

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**Significance of chapter**

In this study, we confirmed that ethanol and nicotine can induce hepatic CYP2E1 protein and activity in primates. Furthermore, ethanol and nicotine-mediated induction of CYP2E1 was related to changes in in vivo CZN disposition. We also demonstrated that the induction of
hepatic CYP2E1 by ethanol self-administration was dose dependent, and that combined exposure to ethanol and nicotine resulted in a greater magnitude of hepatic CYP2E1 induction compared to either ethanol or nicotine exposure alone. The induction of hepatic CYP2E1 can have a wide range of clinical consequences. Of particular concern is the increased activation of toxins and pro-carcinogens as well as the generation of ROS that could occur as a result of CYP2E1 induction. Our results suggest that individuals who are exposed to ethanol and nicotine, particular in combination, may be at higher risk for developing certain toxicities, cancer and diseases.
Chapter 2: Differential effects of nicotine treatment and ethanol self-administration on CYP2A6, CYP2B6 and nicotine pharmacokinetics in African green monkeys

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Dr. Rachel F. Tyndale, Dr. Sharon Miksys, Dr. Roberta Palmour and Charmaine Ferguson contributed to the experimental design. Dr. Sharon Miksys and Dr. Roberta Palmour conducted the animal study. Charmaine Ferguson conducted all the in vitro experiments, performed the data analysis, and wrote the manuscript. Dr. Bin Zhao performed all HPLC procedures for coumarin, 7-hydroxycoumarin, bupropion, hydroxybupropion, nicotine and cotinine quantification. Ewa Hoffman assisted with CYP2A6 and CYP2B6 mRNA quantification. Dr. Rachel F. Tyndale helped with writing and editing the manuscript.

Abstract

In primates, nicotine is metabolically inactivated in the liver by CYP2A6 and possibly CYP2B6. Changes in the levels of these two enzymes may impact nicotine pharmacokinetics and influence smoking behaviours. This study investigated the independent and combined effects of ethanol self-administration and nicotine treatment (0.5 mg/kg bid s.c.) on hepatic CYP2A6 and CYP2B6 levels (mRNA, protein and enzymatic activity), in vitro nicotine metabolism and in vivo nicotine pharmacokinetics in monkeys. CYP2A6 mRNA and protein levels, and in vitro coumarin (selective CYP2A6 substrate) and nicotine metabolism were decreased by nicotine treatment but unaffected by ethanol. CYP2B6 protein levels and in vitro bupropion (selective CYP2B6 substrate) metabolism were increased by ethanol but unaffected by nicotine treatment; CYP2B6 mRNA levels were unaltered by either treatment. Combined ethanol and nicotine exposure decreased CYP2A6 mRNA and protein levels, as well as in vitro coumarin and nicotine metabolism, and increased CYP2B6 protein levels and in vitro bupropion metabolism, with no change in CYP2B6 mRNA levels. Chronic nicotine resulted in higher nicotine plasma levels.
achieved after nicotine administration, consistent with decreased CYP2A6. Ethanol alone, or combined ethanol and nicotine, resulted in lower nicotine plasma levels by a mechanism independent of the change in these enzymes. Thus, nicotine can decrease hepatic CYP2A6 reducing the metabolism of its substrates, including nicotine, while ethanol can increase hepatic CYP2B6 increasing the metabolism of CYP2B6 substrates. In vivo nicotine pharmacokinetics are differentially affected by ethanol and nicotine, but when both drugs are used in combination the effect more closely resembles ethanol alone.

**Introduction**

Nicotine is responsible for the reinforcing effects of cigarette smoking and dependent smokers will adjust their smoking behaviour (i.e., cigarettes/day, inhalation volume, puff frequency) to maintain desired plasma nicotine levels (Scherer, 1999). Therefore, factors that influence nicotine pharmacokinetics, either genetic or environmental (Swan et al., 2009), can have an impact on smoking behaviours and ultimately the risk for certain smoking-related diseases (Liu et al., 2011; Strasser et al., 2011; Wassenaar et al., 2011). Identifying factors that modify nicotine pharmacokinetics is an important step toward understanding smoking behaviours.

In humans, nicotine is mainly eliminated via metabolic inactivation to cotinine in the liver (Benowitz and Jacob, 1994). This reaction is mediated primarily by CYP2A6, with some contribution from CYP2B6 (Messina et al., 1997; Yamazaki et al., 1999). Cotinine is further metabolized to trans-3-hydroxycotinine via a reaction mediated exclusively by CYP2A6 (Nakajima et al., 1996a; Mwenifumbo et al., 2008). Cigarette smoking decreases nicotine metabolism, which is in contrast to the well-known effect of cigarette smoking on accelerating the metabolism of many other drugs (Zevin and Benowitz, 1999). This paradox is presumably
due to the downregulation of hepatic CYP2A6 by nicotine, since chronic nicotine treatment decreased hepatic CYP2A6 mRNA, protein and activity levels in monkeys (Schoedel et al., 2003). CYP2B6 levels and activity are unaffected by smoking and nicotine treatment does not affect hepatic CYP2B6 mRNA, protein or activity levels in monkeys (Schoedel et al., 2003; Hesse et al., 2004). Elevated levels of CYP2A6 and CYP2B6 protein have been reported in livers from alcoholics (Niemelä et al., 2000; Hesse et al., 2004). Chronic ethanol administration induces protein levels of hepatic CYP2A5 (orthologue to human CYP2A6) in mice (Lu et al., 2011) and hepatic CYP2B1 (orthologue to human CYP2B6) in rats (Howard et al., 2001), suggesting that ethanol exposure may be responsible for the higher levels of CYP2A6 and CYP2B6 in alcoholics; however, the induction of these hepatic CYPs by ethanol has not yet been demonstrated in primates.

Despite the large proportion of smokers who regularly consume alcohol, there is limited information about the combined effects of smoking and alcohol consumption on CYP2A6, CYP2B6 and nicotine pharmacokinetics. There have been; however, many studies showing that alcohol consumption can modify smoking behaviours. For example, smokers consume a greater number of cigarettes and inhale more deeply while drinking alcohol (Mintz et al., 1985; Witkiewitz et al., 2011). The association between smoking and alcohol consumption may be due in part to ethanol-induced changes in nicotine pharmacokinetics, possibly involving the induction of CYP2A6 and/or CYP2B6.

The AGM is an ideal animal model for studying hepatic CYP2A6 and CYP2B6 regulation, nicotine metabolism and nicotine pharmacokinetics. The expression, substrate specificity and regulation of AGM CYP2A6 (also referred to as CYP2A26) and CYP2B6 are similar to their respective human orthogues (Schoedel et al., 2003; Uno et al., 2011). Like
humans, AGM eliminate nicotine mainly via metabolic inactivation to cotinine in the liver, with CYP2A6 having a major role in mediating this reaction and CYP2B6 having a minor contribution (Schoedel et al., 2003). The apparent km value (mean ± SD) for in vitro nicotine metabolism in AGM hepatic microsomes is 29.1 ± 8.6 μM (n= 6) (Schoedel et al., 2003), which is comparable to the apparent km value of 64 ± 32.7 μM (n = 31) in human hepatic microsomes (Messina et al., 1997). Consistent with these similarities, the plasma half-life for nicotine ranges from 1-4 hrs in both AGM and humans (Hukkanen et al., 2005; Lee et al., 2006a). AGM are also an important model for complex human behaviours and are routinely used in alcohol research because they will voluntarily self-administer alcohol at levels comparable to human consumption (Ervin et al., 1990).

In this study we characterized the effects of chronic nicotine treatment and ethanol self-administration, alone and in combination, on hepatic CYP2A6 and CYP2B6 levels, in vitro nicotine metabolism and in vivo nicotine pharmacokinetics in AGM. First, we showed that nicotine treatment can decrease hepatic CYP2A6 levels and that ethanol self-administration can increase hepatic CYP2B6 levels. Second, we demonstrated that the metabolism of nicotine to cotinine in monkey liver microsomes is mediated primarily by CYP2A6 and is reduced by nicotine treatment but unaffected by ethanol exposure. Lastly, we showed that exposure to ethanol and nicotine, either alone or in combination, can modify in vivo nicotine pharmacokinetics.

**Methods**

**Animals.** Adult male African green monkeys (vervets, Chlorocebus sabeus) were housed outdoors in social groups at the Behavioural Sciences Foundation (St. Kitts). The study timeline
is shown in Figure 16 and has been previously described in detail (Ferguson et al., 2011). Briefly, the first 14 days of the study consisted of an ethanol preference screening phase, where monkeys were allowed to self-administer 10% v/v ethanol in 0.5% w/v sucrose solution for 4 h/day. Forty monkeys that voluntarily consumed more than 1 g of ethanol/kg per day were randomized into four groups based on daily ethanol consumption (n = 10/group): a no-drug control group, an ethanol-only group, a nicotine-only group and an ethanol + nicotine group. The following 14 days (days 15–28) consisted of a washout period, during which monkeys were not exposed to any ethanol or nicotine. From days 29 to 42, monkeys in the ethanol-only and ethanol + nicotine groups were allowed to self-administer 10% ethanol in 0.5% sucrose solution for 4 h/day, whereas the other groups consumed 0.5% sucrose solution on the same schedule. From days 43 to 63, in addition to ethanol (or sucrose), monkeys in the nicotine-only and ethanol + nicotine groups were given subcutaneous injections of nicotine bitartrate (milligram base in saline, pH 7.0, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) twice daily at a dose of 0.05 mg/kg on day 43, 0.1 mg/kg on day 44, 0.25 mg/kg on day 45, and 0.5 mg/kg for subsequent days. The first injection was given 30 min before the ethanol (or sucrose) access period and the second injection was given 10 h later. Monkeys in the ethanol-only and control groups were given saline injections (as a vehicle control for nicotine bitartrate) on the same schedule. On day 50, nicotine treatment and ethanol access were suspended to conduct pharmacokinetic testing. Throughout the study, monkeys were fed standard rations of Purina monkey chow supplemented with fresh fruit and vegetables twice a day and drinking water was available ad libitum. Body weights at the start of the study (average = 5.9 ± 0.5 kg) were not significantly different from body weights at sacrifice (average = 5.7 ± 0.5 kg) (paired t test, p > 0.05). There were no significant differences in body weights among groups at the start of the study (one-way
ANOVA, $F[3, 36] = 0.3784, p > 0.05$) or at sacrifice (one-way ANOVA, $F[3, 36] = 1.860, p > 0.05$). Animals were sacrificed by exsanguination via the femoral artery under ketamine anesthesia, and livers were immediately dissected and flash-frozen in liquid nitrogen and stored at -80°C until further use. The experimental protocol was reviewed and approved by the Institutional Review Board of the Behavioural Sciences Foundation and the University of Toronto Animal Care Committee. All the procedures were conducted in accordance with the guidelines of Declaration of Helsinki and the Canadian Council on Animal Care.
Monkeys were assessed for ethanol preference and then randomized into four study groups (n =10/group) based on daily ethanol consumption. The groups consisted of a no-drug control group, an ethanol-only group, a nicotine-only group and an ethanol + nicotine group. *In vivo* nicotine pharmacokinetic (PK) testing was performed pre-drug administration on day 22 (during the washout period) and again post-drug administration on day 50. Nicotine treatment and ethanol access were suspended on day 50 to allow for pharmacokinetic testing. Monkeys were sacrificed on day 63.
**Microsomal membrane preparation.** Monkey liver tissue was homogenized in 100 mM Tris, 0.1 mM ethylenediaminetetraacetic acid, 0.1 mM dithiothreitol, and 0.32 M sucrose (pH 7.4) for immunoblotting or in 1.15% w/v KCl for *in vitro* metabolism assays and then centrifuged at 12,500g for 30 min at 4°C. The supernatant was then centrifuged at 110,000 g for 90 min at 4°C, and the pellet was resuspended in 100 mM Tris, 0.1 mM ethylenediaminetetraacetic acid, 0.1 mM dithiothreitol, 1.15% w/v KCl, and 20% v/v glycerol for immunoblotting or 1.15% w/v KCl for *in vitro* metabolism assays. The protein content of liver microsomes was assayed with the Bradford (1976) technique using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Microsomes were stored at -80°C until further use.

**Immunoblotting.** Monkey liver microsomal protein was serially diluted to generate a standard curve and to establish the linear detection range for the immunoblotting assays (Figure 17). Liver microsomal proteins (10 μg for the CYP2A6 assay and 5 μg for the CYP2B6 assay) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 8% separating gels). A portion of each gel was stained with Coomassie Blue R-250 to ensure equal loading of protein among lanes and the remaining gel was transferred overnight onto nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA). To detect CYP2A6 and CYP2B6 protein, the membranes were first blocked with 1% skim milk in 50 mM Tris-buffered saline (TBS) containing 0.1% w/v bovine serum albumin and 0.1% v/v Triton X-100 for 1 h. Membranes were then incubated with either polyclonal anti-human CYP2A6 antibody (Fitzgerald Industries, Acton, MA, USA) or anti-human CYP2B6 antibody (Fitzgerald Industries, Acton, MA, USA) diluted 1:2000 for 2 h, followed by three 5 min washes with TBS containing 0.1% v/v Triton X-
The membranes were then blocked again with the initial blocking solution for 1 h and incubated with peroxidase-conjugated rabbit anti-sheep antibody (Millipore Corporation, Bilerica, MA, USA) diluted 1:5000 for 1 h, followed by three 5 min washes with TBS containing 0.1% v/v Triton X-100. Proteins were visualized using chemiluminescence (Thermo Fisher Scientific, Mississauga, ON, Canada) followed by exposure to autoradiographic film (Ultident, St Laurent, QC, Canada).
Figure 17. Detection of CYP2A6 and CYP2B6 protein in monkey liver microsomes is linear.
A dilution curve of monkey hepatic microsomal protein showed linear detection of (A) CYP2A6 from 0-15 µg and (B) CYP2B6 from 0-10 µg. For the CYP2A6 immunoblotting assay, 10 µg of microsomal protein was used and for the CYP2B6 assay, 5 µg of microsomal protein was used.
**In vitro nicotine c-oxidation assay.** Nicotine c-oxidation (NCO) was assayed as previously described (Schoedel et al., 2003). For the NCO chemical inhibition assays, pooled samples containing equal amounts of microsomal protein from all monkeys in a study group were used. The reaction mixtures were pre-incubated for 15 min with chemical inhibitors. The inhibitor concentrations were approximately equal to Ki and 10-times higher than Ki for the target cytochromes P450 in human liver microsomes: Pilocarpine (CYP2A6, 4 and 40 μM, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) (Zhang et al., 2001), C8-Xanthate (CYP2B6, 1 and 10 μM, Toronto research chemicals, Toronto, ON, Canada) (Bourrié et al., 1996) and quinidine (CYP2D6, 0.4 and 4 μM, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) (Bourrié et al., 1996). Chemically inhibited NCO was compared with reactions performed in the presence of appropriate vehicle controls but not chemical inhibitors. Samples were then analyzed for nicotine and cotinine concentrations.

**In vivo nicotine and cotinine plasma assessments.** To determine *in vivo* kinetic parameters for nicotine and cotinine, on days 22 and 50 monkeys were anesthetized with ketamine and injected with 0.1 mg/kg nicotine s.c. (milligram base in saline, pH 7.0, Sigma-Aldrich Canada Ltd). Blood samples (6 ml) were drawn at baseline and at 10, 20, 30, 60, 120, 240 and 360 min after the injection; due to the need for continuous anesthesia, latter sample collection times were not feasible. The blood samples were centrifuged, and the plasma was removed and frozen at -20°C for subsequent drug analyses. Total nicotine and cotinine was measured after deconjugation by an overnight incubation with β-glucuronidase (15 mg/ml, 250 μl per 0.5 ml of plasma) at 37°C.
Nicotine and cotinine measurements. The method for nicotine and cotinine measurement was adapted from a previous study (Siu et al., 2006). Briefly, samples (either plasma or reaction mixtures from the *in vitro* NCO assays) were extracted with dichloromethane and the organic phase was dried under nitrogen. Samples were reconstituted with 105 μl of 0.01 M HCl and 90 μl of each sample was analyzed by HPLC with UV detection (260 nm). Separation of nicotine and cotinine was achieved using ZORBAX Bonus-RP column (5 μm, 150x4.6 mm; Agilent Technologies, Mississauga, ON, Canada) and a mobile phase consisting of acetonitrile/potassium phosphate buffer (10:90 v/v, pH 5.07) containing 3.3 mM heptane sulfonic acid and 0.5% triethylamine. The separation was performed with a flow rate of 0.9 ml/min. Nicotine and cotinine sample concentrations were determined from standard curves. The quantitation limits were 5 ng/ml for nicotine and 12.5 ng/ml for cotinine. Cotinine concentrations were adjusted to account for baseline plasma cotinine present in monkeys from the nicotine-only and the ethanol + nicotine groups using the following equation: \( C_{\text{adjusted}} = C_t - (C_0 \times e^{kt}) \), where \( C_{\text{adjusted}} \) = baseline adjusted cotinine concentration, \( C_t = \) the actual cotinine plasma concentration measured at time \( t \), \( C_0 = \) concentration of cotinine immediately prior to nicotine dosing, \( k = \) elimination rate constant, and \( t = \) the post-dosing interval at which \( C_t \) was sampled.

*In vitro* coumarin hydroxylation assay and 7-OH-coumarin measurement. The methods used for the *in vitro* coumarin hydroxylation assays and for the measurement of coumarin and 7-OH-coumarin were adapted from previous studies (O'Kennedy, 1996; Li et al., 1997). Monkey hepatic microsomal protein (0.25 mg/ml) was mixed with 50 mM Tris buffer (pH 7.6) and 1.0 mM NADPH. After a 2 min pre-incubation, reactions were initiated by the addition of coumarin (30 μM, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) making the final volume 200 μl.
Incubations were for 5 min at 37°C and 50 µl acetonitrile was added to stop the reaction. Trichloroacetic acid (10 µl, 20% v/v) and the internal standard 4-hydroxycoumarin (20 µl, 1 mg/ml) were added to the reaction mixture, which was then vortexed and spun at 16500 g for 10 min. A 100 µl aliquot of each sample was analyzed by HPLC with UV detection (315 nm). Separation of 7-OH-coumarin was achieved using ZORBAX SB C18 column (5 µm 250 x 4.6 mm, Agilent Technologies, Mississauga, ON, Canada) and a mobile phase consisting of acetonitrile, water and acetic acid (25:75:0.1, v/v). The separation was performed with a flow rate of 1 ml/min. The concentration of 7-OH-coumarin was determined from a standard curve. The quantitation limit for 7-OH-coumarin was 25 ng/ml.

**In vitro bupropion hydroxylation assay and hydroxybupropion measurement.** The methods used for the *in vitro* bupropion hydroxylation assays and for the measurement of hydroxybupropion were adapted from a previous study (Hesse et al., 2000; Loboz et al., 2005). Monkey hepatic microsomal protein (0.25 mg/ml) was mixed with 50 mM Tris buffer (pH 7.6) and 1.0 mM NADPH. After pre-incubation for 2 min, the reactions were initiated by the addition of bupropion (300 µM, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) making the final volume 250 µl. Incubations were for 20 min at 37°C and sodium carbonate (100 µl, 20% v/v) was added to stop the reaction. Timolol (25 µl, 100 µg/ml) was added as an internal standard and the mixture was vortexed and spun at 16500 g for 20 min. A 100 µl aliquot of each sample was analyzed by HPLC with UV detection (210 nm). Separation of hydroxybupropion was achieved using ZORBAX Bonus-RP column (250 x 4.6 mm, 5 µM, Agilent Technologies, Mississauga, ON) and a mobile phase consisting methanol and 0.05 M phosphate buffer (pH = 5.8, 45:55 v/v). The separation was performed with a flow rate of 1 ml/min. The concentration
of hydroxybupropion was determined from a standard curve. The quantitation limit for hydroxybupropion was 10 ng/ml.

**Isolation, cDNA synthesis, and mRNA quantification.** Liver tissue (50-100 mg) was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated according to the TRIzol reagent protocol. RNA concentrations were determined spectrophotometrically and total RNA integrity was confirmed by electrophoresis on a 1.2% agarose gel (Onbio, Inc., Richmond Hill, ON, Canada) stained with ethidium bromide. Total mRNA from one animal from the nicotine-only group was not included in our analysis due to poor RNA integrity. cDNA was synthesized using 1 μg of total RNA, random hexamers (Invitrogen), RiboLock RNase inhibitor (Fermentas, Burlington, ON, Canada) and MMLV Reverse Transcriptase (Invitrogen) according to protocols provided by Invitrogen. Primers for real-time PCR amplification of CYP2A6, CYP2B6 and β-actin were as follows: CYP2A6 forward primer (CYP2A6ex1), 5’-TGGCAGCAGAGGACAGCAA-3’; CYP2A6 reverse primer (CYP2A6ex2), 5’-GCCTCCCTGACGGCATCATA-3’; CYP2B6 forward primer (CYP2B6ex2), 5’-TATGGGGACGTCTTCACA-3’; CYP2B6 reverse primer (CYP2B6ex3), 5’-ATCAGACACTGAGCCTCC-3’; β-actin forward primer (ACTBex3), 5’-CAGAGCAGAGGACAGCAA-3’; and β-actin reverse primer (ACTBex4), 5’-GGTGTCATGACGAGGACAGCAA-3’. The sequences for AGM CYP2A6 and CYP2B6 are not known; primer specificity was based on the CYP2A6 and CYP2B6 sequences from human and rhesus macaque (Macaca mulatta). Amplification and fluorescence detection were performed using the Applied Biosystems Vii6A7 Real-Time PCR system (Invitrogen). The real-time PCR amplification mixture (20 μl) contained 1 μl of synthesized cDNA, 10 μl of 2X Fast SYBR-Green Mix
(Invitrogen), and 0.3 μM concentrations of each primer. Cycling conditions consisted of an initial activation of AmpliTaq Fast DNA polymerase followed by 40 cycles of denaturing (95°C for 15 s) and annealing/extension (58°C for 20 s). Dilutions of monkey and human cDNA were used to determine the range of log-linear detection. CYP2A6 and CYP2B6 mRNA levels were normalized to β-actin mRNA using the comparative CT method for relative quantification as described by the manufacturer (Real-Time PCR Chemistry Guide; Invitrogen).

**Data analysis.** In vivo pharmacokinetics were derived using non-compartmental analysis. Elimination half-life was estimated by the terminal slope of the concentration vs. time curve. The area under the concentration time curve from 0-6h (AUC\(_{0-6h}\)) was calculated using the trapezoidal rule and the area under the concentration curve from 0-infinity (AUC\(_{0-\infty}\)) was calculated using the equation AUC\(_{0-\infty}\) = AUC\(_{0-6h}\) + C\(_{6h}\)/k, where C\(_{6h}\) is the concentration at 6 hr post-nicotine dosing and k is the elimination rate constant. Apparent volume of distribution was estimated using the equation: [Nicotine dose/AUC\(_{0-\infty}\)] x 1/half-life. The effect of nicotine and ethanol on hepatic CYP2A6 and CYP2B6 protein, in vitro activity and mRNA were assessed using a two-way ANOVA (nicotine treatment X ethanol self-administration), followed by a one-way ANOVA and Least Significant Difference post hoc test. Paired Student’s t tests were used to assess differences in in vivo nicotine and cotinine pharmacokinetic parameters measured pre- and post-drug administration. Statistical significance was set at p < 0.05.
Results

Monkeys voluntarily self-administered alcohol

The amount of alcohol consumed by the monkeys in this study has previously been described (Ferguson et al., 2011). Briefly, average daily consumption of 10% ethanol in sucrose solution during the 4hr alcohol access session was 38.12 ± 7.8 ml/kg (3.0 g ethanol/kg). There was no significant difference in mean ethanol consumption between the ethanol-only and the ethanol + nicotine groups, indicating no effect of nicotine treatment on voluntary alcohol consumption (Ferguson et al., 2011).

Nicotine and ethanol alter hepatic levels of CYP2A6 and CYP2B6 respectively

There was a significant main effect of nicotine treatment on hepatic CYP2A6 protein (F[1, 36] = 23.73; p < 0.001; Figure 18A), in vitro activity (F[1, 36] = 27.18; p < 0.001; Figure 18C) and mRNA levels (F[1, 35] = 13.2; p < 0.001; Figure 18E). Compared to the control group, monkeys in the nicotine-only and nicotine + ethanol groups had a 40% (p < 0.01) and 47% (p < 0.05) decrease in hepatic CYP2A6 protein, respectively (Figure 18A). CYP2A6 protein levels in the two nicotine-treated groups were also significantly lower compared to the ethanol-only group (Figure 18A). Hepatic CYP2A6 in vitro activity, measured by the rate of coumarin metabolism to 7-OH-coumarin in monkey liver microsomes, showed a pattern of reduction similar to the CYP2A6 protein levels, with a 55% (p < 0.05) and 35% (p < 0.05) decrease in activity in the nicotine-only and ethanol + nicotine groups, respectively (Figure 18C). Hepatic CYP2A6 mRNA was reduced by 46% (p < 0.01) in the nicotine-only group and 37% (p < 0.05) in the nicotine + ethanol group compared to the control group (Figure 18E). Ethanol self-administration did not significantly affect hepatic CYP2A6 protein (F[1, 36] = 0.02; p = 0.9;
Figure 18A), activity (F[1, 36] = 0.16; p = 0.76; Figure 18C) or mRNA levels (F[1, 35] = 0.16; p = 0.17; Figure 18E). There was no interaction effect between ethanol and nicotine on hepatic CYP2A6 protein (F[1, 36] = 0.39; p = 0.89; Figure19A) or mRNA levels (F[1, 35] = 1.93; p = 0.17; Figure 18E). However, a significant drug interaction on CYP2A6 in vitro activity was observed (F[1, 36] = 13.02; p < 0.01; Figure 18C). This may be a spurious finding, since post-hoc testing revealed no significant difference in in vitro CYP2A6 activity between the nicotine-only group and the ethanol + nicotine group.
Figure 18. CYP2A6 and CYP2B6 are altered by nicotine and ethanol, respectively.
Mean CYP2A6 (A) and CYP2B6 (B) hepatic protein levels (n=10/group). Representative blots for CYP2A6 and CYP2B6 are shown with Coomassie blue staining to indicate equal protein loading among lanes (n=4 shown of 10/group analyzed). C, mean velocity of 7-OH-coumarin formation in monkey liver microsomes (n=10/group). D, mean velocity of hydroxybupropion formation in monkey liver microsomes (n=10/group). Mean hepatic CYP2A6 (E) and CYP2B6 (F) mRNA levels normalized to β-actin (n=9 for nicotine-only group and n = 10 for all other groups). For all graphs: *p<0.05 compared to control group, †p<0.05 compared to ethanol-only group, #p<0.05 compared to nicotine-only group.
There was also significant main effect of ethanol self-administration on hepatic CYP2B6 protein (F[1, 36] = 18.81; p < 0.001; Figure 18B) and in vitro activity (F[1, 36] = 12.16; p = 0.001; Figure 18D). Compared to the control group, monkeys in the ethanol-only and the ethanol + nicotine groups had a 1.96-fold (p < 0.01) and 1.73-fold (p < 0.05) increase in hepatic CYP2B6 protein, respectively. CYP2B6 protein levels in the ethanol-consuming groups were also significantly higher compared to the nicotine-only group (Figure 18B). Hepatic CYP2B activity, measured by the rate of bupropion metabolism to hydroxybupropion in monkey liver microsomes, showed a pattern of increase similar to the CYP2B6 protein levels, and with a 1.26-fold (p > 0.05) and 1.51-fold (p < 0.05) increase in activity in the ethanol-only and ethanol + nicotine groups, respectively. Ethanol-self administration did not significantly affect CYP2B6 mRNA levels in the liver (F[1, 35] = 0.13, p = 0.7; Figure 18F). As a positive control for transcriptional induction of CYP2B6, we used hepatic mRNA from AGMs treated with phenobarbital, a known inducer of CYP2B6 mRNA and protein (Lee et al., 2006a). A 6.5-fold increased in mRNA was detected in the livers from phenobarbital-treated monkeys compared to untreated control monkeys, indicating that phenobarbital, but not ethanol, induces CYP2B6 via a transcriptional mechanism. Nicotine treatment did not significantly affect hepatic CYP2B6 protein (F[1, 36] = 0.0003, p = 0.99, Figure 18B), in vitro activity (F[1, 36] = 0.95; p = 0.4; Figure 18D) or mRNA levels (F[1, 35] = 2.86; p = 0.1; Figure 18F). There was no interaction effect between ethanol and nicotine on hepatic CYP2B6 protein (F[1, 36] = 2.10; p = 0.99; Figure 18B), in vitro activity (F[1, 36] = 1.54; p = 0.2; Figure 2D) or mRNA (F[1, 35] = 0.15; p = 0.70; Figure 18F).
In vitro nicotine c-oxidation is decreased by nicotine treatment but unaffected by ethanol self-administration

The rate of NCO in monkey liver microsomes was assessed at two nicotine concentrations: 30 µM (= K_m for NCO) and 300 µM (= V_max for NCO) (Schoedel et al., 2003). At the 30 µM concentration, there was a significant main effect of nicotine treatment on the rate of in vitro NCO (F[1, 36] = 14.08;  p < 0.001), with a 47% (p < 0.05) decrease in the nicotine-only group and a 32% (p > 0.05) decrease in the nicotine + ethanol group compared to the control group (Figure 19A). Similarly, at the 300 µM substrate concentration, there was also a significant effect of nicotine on the rate of in vitro NCO (F[1, 36] = 11.12;  p < 0.001), with a 42% (p < 0.05) decrease in the nicotine-only group and a 20% (p > 0.05) decrease in the nicotine + ethanol group compared to the control group (Figure 19B). Ethanol did not have a significant effect on NCO at either the 30 µM (F[1, 36] = 2.25;  p = 0.14; Figure 19A) or 300 µM nicotine concentrations (F[1, 36] = 3.13;  p = 0.085; Figure 19B). There was no interaction effect between ethanol and nicotine on the rate of NCO at 30 µM (F[1, 36] < 0.001;  p = 0.99) or at 300 µM (F[1, 36] = 0.05;  p = 0.82).
Figure 19. NCO is decreased by nicotine treatment but unaffected by ethanol self-administration.

*In vitro* NCO in liver microsomes (mean + SE, n = 10/group) at two nicotine concentrations: (A) 30 μM (≈ Km for NCO) or (B) 300 μM (≈ Vmax for NCO). For all graphs: *p<0.05 compared to control group, †p<0.05 compared to ethanol-only group.
Nicotine c-oxidation is primarily mediated by CYP2A6 in monkey liver

Pilocarpine, a selective chemical inhibitor of CYP2A6, strongly inhibited NCO in monkey liver microsomes from all four study groups to a similar extent. At 30 μM nicotine (≈ Km for NCO), a concentration of pilocarpine equivalent to Ki decreased NCO by 68-74% and a concentration 10-times higher than Ki, decreased NCO by approximately 94% (Figure 20A). Results were similar at 300 μM nicotine (≈ Vmax for NCO) with 36-44% inhibition at a concentration of pilocarpine equivalent to Ki and with 75-79% NCO inhibition at 10-times Ki (Figure 20B). The selective CYP2B6 inhibitor C8-xanthate used at a concentration equivalent to Ki had little effect on NCO at 30μM or 300 μM nicotine in monkey liver microsomes from all four groups. At 10-times Ki, C8-xanthate decreased NCO by 35-38% at 30 μM nicotine (Figure 20A) and 18-27% at 300 μM nicotine (Figure 20B). Quinidine, a selective CYP2D6 inhibitor, was used as a negative control and did not inhibit NCO at any concentration of nicotine or inhibitor (Figure 20).
Figure 20. NCO is mediated primarily by CYP2A6 in monkey liver.
Assays were performed in duplicate at (A) 30 µM or (B) 300 µM nicotine (n=10 per pooled sample). Percent NCO formation with chemical inhibitors at concentrations equivalent to human Ki or 10-times Ki [pilocarpine (4 µM and 40 µM), C8-Xanthate (1 µM and 10 µM) and quinidine (0.5 µM and 5 µM)]. Values are expressed as percent of vehicle control.
There was a significant positive correlation between hepatic CYP2A6 protein levels and *in vitro* NCO at both 30 µM (R = 0.64, p<0.001; Figure 21A) and 300 µM nicotine concentrations (R = 0.52, p < 0.001; Figure 21C). Hepatic CYP2B6 protein levels did not correlate with NCO at either 30 µM (R = -0.20, p = 0.13; Figure 21B) or 300 µM (R = -0.09, p = 0.3; Figure 21D) nicotine concentrations.
Figure 21. The rate of *in vitro* nicotine c-oxidation significantly correlated with CYP2A6 but not CYP2B6 protein levels. There was a positive correlation between CYP2A6 protein levels and NCO at 30 μM (A) and 300 μM (C) nicotine. There was no significant correlation between CYP2B6 protein and NCO at 30 μM (B) and 300 μM (D).
**Ethanol and nicotine modify *in vivo* nicotine pharmacokinetics**

Like humans, monkeys have substantial interindividual variation in nicotine pharmacokinetics (see Figure 22, control day 22) hence a within-animal design was used to assess changes in *in vivo* nicotine disposition. After chronic nicotine treatment, monkeys in the nicotine-only group had a 1.39-fold increase in nicotine half-life (*p* < 0.01), a 1.50-fold increase in nicotine area under the curve from 0-6 h (AUC$_{0-6h}$) (*p* < 0.05), and a 1.66 increased in nicotine area under the curve extrapolated to infinity (AUC$_{0-infinity}$) (*p* < 0.05), (Table 14, Figure 22 and 23). After chronic ethanol self-administration, monkeys in the ethanol-only group had a 72% reduction in the maximal concentration of nicotine (C$_{max}$) (*p* < 0.05), a 52% reduction in nicotine AUC$_{0-6h}$ (*p* < 0.05) and 50% reduction in nicotine AUC$_{0-infinity}$ (*p* < 0.05), (Table 14, Figure 22 and 23).

Combined ethanol self-administration and nicotine treatment increased nicotine half-life by 1.26-fold, decreased the nicotine C$_{max}$ by 76% (*p* < 0.05), AUC$_{0-6h}$ by 62% (*p* < 0.01) and AUC$_{0-infinity}$ by 54% (*p* < 0.01) (Table 14, Figure 22 and 23). Therefore combined ethanol self-administration and nicotine treatment increased nicotine half-life, an effect that is presumably mediated by nicotine treatment, and decreased nicotine AUC and C$_{max}$, an effect that appears to be mediated by ethanol. No significant changes in nicotine kinetic parameters were observed in the control group between pharmacokinetic challenge days (Table 14, Figure 22 and 23).
Figure 22. Ethanol self-administration and nicotine treatment alter in vivo nicotine disposition.
Lines represent the change in plasma nicotine $\text{AUC}_{0-6\text{hr}}$ for individual monkeys from pre-drug administration (day 22) to post-drug administration (day 50). Diamonds with error bars represent mean $\text{AUC}_{0-6\text{hr}} \pm \text{SE}$ for the group pre- and post-drug administration. *p<0.05 compared to same group of monkeys pre-drug administration.

Table 14. Nicotine pharmacokinetic parameters (mean ± SE, n = 10 per group)

<table>
<thead>
<tr>
<th>Nicotine Parameter</th>
<th>Control</th>
<th>Ethanol-only</th>
<th>Nicotine-only</th>
<th>Ethanol + Nicotine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 22</td>
<td>Day 50</td>
<td>Pre-</td>
<td>Post-</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>36.0 ± 15.0</td>
<td>36.9 ± 15.3</td>
<td>51.9 ± 17.1</td>
<td>14.6 ± 2.4*</td>
</tr>
<tr>
<td>Elimination half-life (h)</td>
<td>3.6 ± 0.9</td>
<td>3.2 ± 0.4</td>
<td>2.09 ± 0.3</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>$\text{AUC}_{0-6\text{h}}$ (ng/ml.h)</td>
<td>79.5 ± 29.0</td>
<td>95.3 ± 26.8</td>
<td>66.1 ± 15.5</td>
<td>31.8 ± 4.2*</td>
</tr>
<tr>
<td>$\text{AUC}_{0-\infty}$ (ng/ml.h)</td>
<td>99.2 ± 31.9</td>
<td>121.9 ± 27.3</td>
<td>73.7 ± 16.5</td>
<td>36.9 ± 6.4*</td>
</tr>
</tbody>
</table>

No significant difference in pre-drug administration parameters between groups

Pharmacokinetic parameters were calculated for each animal individually and then average

*p<0.05, compared to pre-drug administration from the same group

AUC; Area under the curve, Cmax; Maximal concentration
Figure 23. The nicotine concentration vs. time curves after 0.1 mg/kg s.c. nicotine injection for monkeys pre-treatment (day 22) and post-treatment (day 50).
For the control group each point represents the mean nicotine concentration for 9 monkeys. For all other groups each point represents the mean for 10 monkeys.
After chronic nicotine treatment, monkeys in the nicotine-only group had a 2.48-fold increase in cotinine AUC (p < 0.01) and a 2.52-fold increase in cotinine C\textsubscript{max} (p < 0.01) (Table 15, Figure 24). Similarly, combined ethanol self-administration and nicotine treatment increased cotinine AUC by 1.89-fold (p < 0.001) and increased cotinine C\textsubscript{max} by 1.82-fold (p < 0.01) (Table 15, Figure 24). No significant changes in cotinine kinetic parameters were observed in the control or ethanol-only groups between pharmacokinetic challenge days (Table 15, Figure 24). Therefore, the effect of combined ethanol and nicotine treatment on cotinine pharmacokinetics appears to be an effect mediated by nicotine treatment, not by ethanol.
Table 15. Cotinine pharmacokinetic parameters (mean ± SE, n =10 per group)

<table>
<thead>
<tr>
<th>Cotinine Parameter</th>
<th>Control Day 22</th>
<th>Ethanol-only Day 22</th>
<th>Nicotine-only Day 22</th>
<th>Ethanol + Nicotine Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre -</td>
<td>Post -</td>
<td>Pre -</td>
<td>Post -</td>
</tr>
<tr>
<td>AUC_{0-6h} (ng/ml.h)</td>
<td>240 ± 56.6</td>
<td>256.5 ± 22.4</td>
<td>162.9 ± 24.4</td>
<td>404.8 ± 5.5*</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>57.4 ± 10.9</td>
<td>53.8 ± 4.5</td>
<td>41.8 ± 4.5</td>
<td>105.2 ± 16.4*</td>
</tr>
<tr>
<td></td>
<td>47.4 ± 8.0</td>
<td>59.9 ± 12.6</td>
<td>42.5 ± 6.7</td>
<td>77.2 ± 10.8*</td>
</tr>
</tbody>
</table>

No significant difference in pre-drug administration parameters between groups

Pharmacokinetic parameters were calculated for each animal individually and then average

*p<0.05, compared to pre-drug administration from the same group

*Cotinine measurements were adjusted to account for baseline plasma cotinine

AUC; Area under the curve, Cmax; Maximal concentration

Figure 24. The cotinine concentration vs. time curves after a 0.1 mg/kg s.c. nicotine injection of monkeys pre-treatment (day22) and post-treatment (day 50).
Each point represents the mean cotinine concentration for 10 monkeys.
Discussion

Nicotine treatment and/or ethanol exposure did not significantly change the relative proportion of NCO mediated by hepatic CYP2A6 and CYP2B6. For example, monkeys exposed to nicotine and ethanol in combination had approximately a 50% reduction in hepatic CYP2A6 protein and 2-fold increase in hepatic CYP2B6 protein. However NCO in these animals was still mediated primarily by CYP2A6 with very little contribution from CYP2B6. These results argue against the concept that CYP2B6 plays a greater role in nicotine metabolism among individuals with reduced CYP2A6 activity (Ring et al., 2007). CYP2A6 protein was significantly correlated with NCO activity, whereas variability in CYP2B6 was not associated with changes in NCO. Similar results have also been shown in human liver microsomes (Koudsi and Tyndale, 2010; Koudsi et al., 2010), suggesting that CYP2A6, but not CYP2B6, hepatic activity is an important factor influencing nicotine metabolism.

Nicotine treatment increased the plasma half-life of nicotine by approximately 1.3-fold in monkeys, suggesting that nicotine is the agent in cigarette smoke responsible for the quantitatively similar decrease in nicotine clearance (12-27%) during smoking compared to non-smoking (Benowitz and Jacob, 1993; 2000). The total daily dose of nicotine administered to the monkeys (1.0 mg/kg/day) is similar to the average amount of nicotine acquired from smoking (0.2 to 1.1 mg/kg) (Benowitz and Jacob, 1984). This dose produced mean nicotine plasma levels of approximately 25 ng/mL for over 16 hrs/day in AGM, which is within the range of nicotine plasma levels achieved by a smoker during waking hours (10-50 ng/mL) (Benowitz NL, 1990). Our nicotine treatment paradigm was shown to downregulate CYP2A6 leading to slower nicotine metabolism and decreased nicotine clearance in vivo. In contrast, the administration of a 42-mg transdermal nicotine patch twice a day for 10 days did not affect nicotine clearance in humans.
(Hukkanen et al., 2009). In this human study, the measurement of hepatic CYP2A6 mRNA and protein was not feasible and the impact of nicotine on in vivo CYP2A6 activity was not assessed. Our study, however, was specifically designed to concurrently investigate the impact of nicotine treatment on CYP2A6 levels, nicotine metabolism and in vivo nicotine disposition.

Nicotine treatment also had a significant impact on cotinine pharmacokinetics, resulting in an increased plasma cotinine AUC after nicotine administration. Cotinine is metabolized to its major metabolite trans-3-OH-cotinine in a reaction mediated exclusively by CYP2A6 (Nakajima et al., 1996a). While nicotine and cotinine are both metabolized by hepatic CYP2A6, cotinine has a much lower hepatic extraction ratio and thus changes in the level of hepatic CYP2A6 will have a greater impact on cotinine clearance compared to nicotine clearance (Hukkanen et al., 2005). Therefore it was expected that the downregulation of hepatic CYP2A6 would decrease the rate of cotinine metabolism to a greater extent than the rate of cotinine formation, resulting in an overall increase in cotinine levels.

The daily amount of alcohol self-administered by the monkeys is comparable to moderate human alcohol consumption (Ferguson et al., 2011). At this level of intake, ethanol did not affect hepatic CYP2A6 protein levels or activity. Our findings are consistent with a human study that showed no association between level of alcohol intake and in vivo CYP2A6 activity in a population of moderate alcohol consumers (Mwenifumbo et al., 2007). It has been proposed that hepatic CYP2A6 may be induced in response to ethanol-mediated oxidative stress and/or liver damage (Lu et al., 2011). Treatment with 100 mM ethanol induced CYP2A6 in a human monocytic cell line and this induction was blocked by pre-treatment with the antioxidant Vitamin C, suggesting a role for oxidative stress in the regulation of CYP2A6 (Jin et al., 2012). This mechanism may contribute to the elevated levels of CYP2A6 found in the livers of alcoholics.
compared to non-alcoholics (Niemelä et al., 2000). However, moderate alcohol consumption, which was modeled in our study, did not induce CYP2A6 mRNA, protein levels or activity.

Ethanol induced hepatic CYP2B6 protein and activity in AGM, suggesting that ethanol exposure is responsible, at least in part, for the higher levels of CYP2B6 in livers from alcoholics compared to non-alcoholics (Hesse et al., 2004). Hepatic CYP2B6 mRNA was not significantly altered by ethanol, indicating a non-transcriptional mechanism of induction at this dose and duration of ethanol exposure. In rats, both transcriptional and non-transcriptional mechanism for the induction of CYP2B6 by ethanol have been reported (Nanji et al., 1994a; Schoedel et al., 2001).

The induction of CYP2B6 by ethanol did not significantly alter in vitro NCO; however, chronic ethanol self-administration substantially decreased nicotine Cmax and AUC by more than 50%. Post-ethanol in vivo nicotine pharmacokinetic testing was performed 24 hrs after the last ethanol self-administration session, at which point blood ethanol levels would be negligible, based on an average ethanol elimination rate of 40-62 mg%/h in AGM (Grant and Bennett, 2003). Therefore, the changes in nicotine pharmacokinetic parameters observed in the ethanol-exposed monkeys are not likely to be due to the acute effects of ethanol. This is consistent with human studies showing no effect of short term ethanol exposure on nicotine pharmacokinetics (Benowitz et al., 1986). We speculate that chronic ethanol exposure may be affecting the distribution kinetics of nicotine. Monkeys that self-administered ethanol had a noticeable but non-significant increase in the apparent volume of distribution (Vd) of nicotine, whereas monkeys in the control and nicotine-only groups showed no change in nicotine Vd over the course of the study. Our estimation of Vd is based on the assumption of 100% nicotine bioavailability from a subcutaneous injection and pseudo-equilibrium conditions (Le Houezec et
al., 1993); however, these assumptions may not be correct. In rats, chronic administration of ethanol increased the Vd of procainamide, a drug that shares structural and chemical similarities with nicotine (Gole and Nagwekar, 1991), consistent with the possibility that ethanol can increase nicotine Vd. The potential effect of ethanol on nicotine Vd may be explained by ethanol’s effect on cell membrane structure and permeability, and epithelial barrier function (Nanji et al., 1994a; Bor et al., 1998; Carrasco et al., 2006; 2007). An increase in nicotine Vd can explain the decrease in nicotine $C_{\text{max}}$ observed in the ethanol-exposed animals, but cannot account for the observed decreased in nicotine $\text{AUC}_{0-\infty}$. This suggests that ethanol is also affecting nicotine clearance, possibility by increasing renal clearance or the efficiency of in vivo metabolic clearance in the liver. Monkeys in the ethanol + nicotine group had a reduction in nicotine AUC after treatment, which was similar to the ethanol-only group but opposite to the nicotine-only group. Therefore, the influence of ethanol on nicotine plasma levels, which is potentially mediated by an increase in nicotine Vd, seems to outweigh the effect of nicotine treatment, which is mediated by a decrease in nicotine metabolism.

A limitation of our study was the duration (6 hours) of pharmacokinetic sampling, which was restricted due to the need for continuous anesthesia. For most animals the ratio of $\text{AUC}_{0.6h} / \text{AUC}_{0-\infty}$ was greater than 0.8, indicating an acceptable proportion of the $\text{AUC}_{0-\infty}$ is captured by the $\text{AUC}_{0.6h}$. However, chronic nicotine treatment significantly increased the plasma half-life of nicotine, which decreased the ratio of $\text{AUC}_{0.6h} / \text{AUC}_{0-\infty}$ to approximately 0.7 for the nicotine-treated animals, potentially reducing the reliability of the estimates of nicotine AUC and half-life for the nicotine-only and nicotine + ethanol groups.

In this study nicotine exposure and ethanol consumption were identified as factors that modify nicotine pharmacokinetics. Nicotine downregulated its own CYP2A6-mediated
metabolism and decreased \textit{in vivo} nicotine clearance, a paradoxical effect. Chronic ethanol consumption substantially decreased the nicotine plasma levels achieved after nicotine administration, an effect that cannot be attributed to altered CYP2A6, CYP2B6 or nicotine metabolism. The lower nicotine plasma levels achieved after ethanol exposure may contribute to the greater number of cigarettes/day smoked by individuals who regularly consume alcohol compared to those who do not (Witkiewitz et al., 2011). Nicotine and ethanol seem to have opposing effects on nicotine pharmacokinetics, but when nicotine and ethanol are presented in combination, the effect of ethanol on nicotine plasma levels dominates. Thus, it is important that researchers and clinician are aware that smokers who regularly consume alcohol may have lower plasma nicotine levels compared to smokers who have the same nicotine intake but rarely consume alcohol. This may also have implications for the efficacy of nicotine replacement therapies.

Both CYP2A6 and CYP2B6 metabolize a variety of compounds other than nicotine; these include clinical drugs, toxicants, pro-carcinogens, drugs of abuse and endogenous molecules (Honkakoski and Negishi, 1997; Mo et al., 2009). The downregulation of CYP2A6 by nicotine, and the induction of CYP2B6 by ethanol in humans could potentially alter the efficacy of clinical drugs, the susceptibility to chemical toxicity and carcinogenesis, and vulnerability to drug abuse.

In conclusion, our findings demonstrate that nicotine treatment can decrease hepatic CYP2A6 levels, ethanol self-administration can induce hepatic CYP2B6 levels, and combined nicotine and ethanol exposure can alter levels of both enzymes respectively. In addition, our results suggest that chronic exposure to nicotine and ethanol, either alone or in combination, can modify \textit{in vivo} nicotine pharmacokinetics in humans.
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Significance of chapter

We have demonstrated for the first time in primates that moderate ethanol self-administration can induce hepatic CYP2B6 protein and activity, but does not affect hepatic CYP2A6. We also confirmed previous reports that nicotine downregulates hepatic CYP2A6 protein and activity, but does not affect hepatic CYP2B6. In addition, we showed that the effects of ethanol and nicotine on hepatic CYP2A6 and CYP2B6 are additive, with combined ethanol and nicotine altering the levels and activity of both these enzymes. Our results suggest that humans who are exposed to ethanol and nicotine may have altered disposition and response to xenobiotics that are metabolized CYP2B6 and CYP2A6 respectively.

We also demonstrated for the first time that chronic exposure of non-human primates to ethanol and nicotine, both alone and in combination, can alter nicotine pharmacokinetics. Of particular interest is our finding that chronic moderate ethanol self-administration decreases the nicotine AUC achieved after a s.c. nicotine injection. Ethanol mediated changes in nicotine pharmacokinetics may be a mechanism by which ethanol consumption can modify smoking behaviours.
Chapter 3: Ethanol self-administration and nicotine treatment induces brain levels of CYP2E1 and CYP2B6 in African green monkeys

Charmaine S. Ferguson, Sharon Miksys, Roberta Palmour and Rachel F. Tyndale

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Dr. Rachel F. Tyndale, Dr. Sharon Miksys, Dr. Roberta Palmour and Charmaine Ferguson contributed to the experimental design. Dr. Sharon Miksys and Dr. Roberta Palmour conducted the animal study. Charmaine Ferguson conducted all the in vitro experiments, performed the data analysis, and wrote the manuscript. Ewa Hoffman assisted with CYP2E1 and CYP2B6 mRNA quantification. Dr. Rachel F. Tyndale helped with writing and editing the manuscript.

Abstract

CYP2B6 and CYP2E1 are enzymes responsible for the metabolism of many centrally acting drugs, toxins and endogenous compounds. Human smokers and alcoholics have elevated levels of CYP2B6 and CYP2E1 in certain brain regions, which may contribute to altered drug efficacy, neurotoxicity and metabolic tolerance. The objective of this study was to determine the effects of ethanol self-administration and nicotine treatment, alone and in combination, on brain CYP2B6 and CYP2E1 levels in monkeys. Monkeys were randomized into four groups (N=10/group): an ethanol-only group, a nicotine-only group, an ethanol + nicotine group and a control (no drug) group. Ethanol (10% alcohol in sucrose solution) was voluntarily self-administered by the monkeys and nicotine was given as subcutaneous injections (0.5 mg/kg bid). Immunocytochemistry revealed induction of both CYP2B6 and CYP2E1 protein in certain brain regions and cells within monkey brain as a result of ethanol self-administration, nicotine treatment and combined exposure to both drugs. Immunoblotting analyses demonstrated CYP2B6 induction by ethanol in the caudate, putamen and cerebellum (1.5-3.2 fold, P<0.05), and CYP2E1 induction by nicotine in the frontal cortex and putamen (1.6-2.0 fold, P<0.05). Combined ethanol and nicotine exposure induced CYP2B6 in the caudate, putamen, thalamus...
and cerebellum (1.4-2.4 fold, P<0.05), and CYP2E1 in the frontal cortex and putamen (1.5-1.8, P<0.05). CYP2B6 and CYP2E1 mRNA levels were unaffected by ethanol or nicotine exposure. In summary, ethanol and nicotine can induce CYP2B6 and CYP2E1 protein in the primate brain, which could potentially result in altered sensitivity to centrally acting drugs and toxins.

**Introduction**

CYPs are responsible for metabolizing a vast array of compounds, including therapeutic drugs, drugs of abuse, toxins and endogenous molecules. The total CYP content in the brain is relatively low compared to the liver (Renaud et al., 2011), so it is unlikely that CYP-mediated metabolism in the brain can substantially influence systemic drug and metabolite levels. However, brain CYPs are functional and highly concentrated in specific regions and cell types in the brain, allowing for a potentially significant impact on metabolism in certain brain microenvironments (Hedlund et al., 2001). The expression of CYPs within certain regions of the brain can increase substantially in response to chemical inducers (Ferguson and Tyndale, 2011). Regional increases in the levels of certain CYPs in the brain can have wide-ranging effects, such as altered susceptibility to neurotoxicity, metabolic tolerance and changes in the efficacy of centrally acting drugs (Ferguson and Tyndale, 2011).

CYP2B is expressed in rat, monkey and human brain (Miksys et al., 2000; 2003; Lee et al., 2006b; Ferguson and Tyndale, 2011). This enzyme can metabolize a wide range of centrally-acting substances including drugs of abuse (e.g., nicotine and 3,4-methylenedioxy-N-methylamphetamine), clinical drugs (e.g., bupropion and propofol), as well as endogenous signaling molecules (e.g., serotonin and testosterone) (Howard et al., 2003; Joshi and Tyndale, 2006a; Mo et al., 2009). The expression of human CYP2B6 was examined in a relatively large
sample of the post-mortem brains from alcoholics and smokers (Lieber, 1997; Miksys et al., 2003). Higher amounts of CYP2B6 protein were detected in the hippocampus, caudate and putamen of alcoholics compared to non-alcoholics, whereas higher CYP2B6 protein levels were detected in the hippocampus, caudate, putamen and cerebellum of smokers compared to non-smokers (Koop, 1992; Miksys et al., 2003). Due to the high prevalence of tobacco and alcohol co-use among the study subjects, it was not possible to independently assess the impact of smoking versus alcohol consumption on brain levels of CYP2B6.

CYP2E1 has also been detected in the rat, monkey and human brain (Howard et al., 2003; Joshi and Tyndale, 2006b; Zhong et al., 2012). This enzyme metabolizes a variety of centrally acting substances including ethanol, clinical drugs (e.g., acetaminophen and halothane), environmental pollutants (e.g., carbon tetrachloride and benzene), as well as endogenous molecules (e.g., arachadonic acid and fatty acids) (Lieber, 1999). Many substrates are neurotoxins or pro-carcinogens that require metabolic bioactivation by CYP2E1 to produce reactive intermediates (Koop, 1992). Even in the absence of a substrate, CYP2E1 can produce high levels of ROS, which can lead to DNA damage, lipid peroxidation and cell death (Caro and Cederbaum, 2004; Renaud et al., 2011). A pilot study was conducted to examine CYP2E1 protein expression in post-mortem brains from alcoholics and smokers (Hedlund et al., 2001; Howard et al., 2003). Using immunocytochemistry, higher amounts of CYP2E1 protein were observed in the hippocampus and cerebellum of alcoholic non-smokers compared to non-alcoholic non-smokers, whereas CYP2E1 protein levels were higher in the frontal cortex, hippocampus and cerebellum of alcoholic smokers compared to alcoholic non-smokers (Howard et al., 2003; Ferguson and Tyndale, 2011). This study suggested that alcoholics and smokers have elevated brain levels of CYP2E1.
A causal relationship between alcohol or tobacco use and higher brain levels of CYP2B6 and CYP2E1 is difficult to demonstrate in humans. However, the effects of ethanol or tobacco constituents on CYP2B6 and CYP2E1 levels in the human brain can be predicted using non-human primates. We investigated the independent and combined effects of ethanol self-administration and nicotine treatment on brain levels of CYP2B6 and CYP2E1 in AGMs. Humans and AGMs are similar with respect to expression, substrate specificity and regulation of CYP2B6 and CYP2E1 (Joshi and Tyndale, 2006b; Lee et al., 2006b). The demonstration of CYP2E1 and CYP2B6 induction in monkey brain would strongly suggest that this induction also occurs in humans.

**Material and methods**

**Animals**

Adult (age 6-8 yrs) male African green monkeys (vervets, *Chlorocebus sabeus*) from the Behavioural Sciences Foundation (St. Kitts) were housed as previously described (Palmour et al., 1998). Monkeys were given standard rations of Purina monkey chow supplemented with fresh fruits and vegetables twice a day, with drinking water available ad libitum.

**Drug treatment**

The study timeline is shown in Figure 25 and has been described previously in detail (Ferguson et al., 2011). Days 1-28 of the study consisted of ethanol preference screening, followed by a washout period. Forty monkeys that consumed more than 1.0 g/kg of ethanol in a 4 h period were randomized into four groups (N=10/group) based on daily ethanol consumption (average ethanol consumption per group = 3.2 ± 1.0 g ethanol/kg): control group, an ethanol-only group, a
nicotine-only group and an ethanol + nicotine group. From day 29 to 42, monkeys in the ethanol-only and ethanol + nicotine groups were allowed to self-administer 10% ethanol in 0.5% sucrose solution for 4 h/day, whereas the other groups self-administered 0.5% sucrose solution on the same schedule.

The self-administration sessions were performed in individual cages equipped with two drinking bottles (500 ml): one bottle contained either ethanol in sucrose solution or sucrose solution alone, the other bottle contained water. On days 43-63 of the study, in addition to ethanol in sucrose (or sucrose alone), monkeys in the nicotine-only and ethanol + nicotine groups were given subcutaneous injections of nicotine bitartrate (milligram nicotine base in saline, pH 7.0; Sigma Aldrich Canada Ltd, Oakville, ON, Canada) twice daily at a dose of 0.05 mg/kg on day 43, 0.1 mg/kg on day 44, 0.25 mg/kg on day 45, and 0.5 mg/kg for subsequent days. Monkeys in the ethanol-only and control groups were given saline injections on the same schedule. Animals were sacrificed under ketamine anesthesia by exsanguination via the femoral artery. The experimental protocol was reviewed and approved by the Institutional Review Board of the Behavioural Sciences Foundation and the University of Toronto Animal Care Committee. All experiments comply with the Animal Research: Reporting of In Vivo Experiments guidelines and the guidelines adopted by the National Institutes of Health guide for the care and use of Laboratory animals.
Figure 25. Overview of study timeline.
After a screening for ethanol preference monkey were randomized into the four study groups (n =10/group) based on daily ethanol consumption. Monkeys that did not self-administer ethanol were allowed to self-administer sucrose solution on the same schedule. Monkeys that did not receive nicotine injections were given saline injections on the same schedule.
**Immunocytochemistry**

Immunocytochemical staining for CYP2B6 and CYP2E1 was performed on frozen brain tissue sections (10 µM) as previously described by (Lee et al., 2006b), except the tissue sections were incubated with either a polyclonal anti-CYP2E1 (1:1000; Fitzgerald Industries, Acton, MA) or a polyclonal anti-CYP2B antibody (1:1000; Fitzgerald Industries), then incubated with a biotinylated anti-rabbit secondary antibody (1:500; Vector Laboratories, Burlingame, CA) for detection. Control sections that were not incubated with primary antibody were processed in parallel. A single observer blinded to treatment visually assessed the intensity of CYP2E1 or CYP2B6 immunoreactivity using a semi-qualitative scoring system: no staining (-), weak staining (+), moderate staining (++), strong staining (+++) and very strong staining (+++). The thalamus was not available for evaluation in the brain tissue that was dissected and processed for immunocytochemical analysis.

**Brain membrane preparation**

Whole membranes were prepared from dissected brain tissue as previously described (Miksys et al., 2002). The protein content of the brain membranes was assessed with the Bradford (1976) technique using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

**Immunoblotting**

Immunoblotting was performed using the same polyclonal anti-CYP2B (Fitzgerald) and polyclonal anti-CYP2E1 antibodies (Fitzgerald) that were used for the immunocytochemical analysis. Brain membrane protein (from either the frontal or parietal cortex of a control monkey) was serially diluted to generate a standard curve and to establish the linear detection range for the
immunoblotting assays, which was 10-80 µg for CYP2B6 detection and 5-60 µg for CYP2E1 detection (Figure 26). The anti-CYP2B antibody detected cDNA-expressed human CYP2B6, but did not cross react with other cDNA-expressed human CYPs (CYP2E1, CYP2A6, CYP1A1, CYP1A2, CYP2C19, CYP2D6, CYP3A4). Likewise, the anti-CYP2E1 antibody detected cDNA-expressed human CYP2E1, but did not cross react with other cDNA-expressed human CYPs (CYP2B6, CYP2A6, CYP1A1, CYP1A2, CYP2C19, CYP2D6, CYP3A4). Brain CYP2B6 and CYP2E1-immunoreactive protein bands co-migrated with human cDNA-expressed CYP2B6 (56 kDa) and CYP2E1 proteins (57 kDa), respectively (Figure 26).

Membrane proteins (20-40 µg) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 8% separating gels), stained with Coomassie Blue R-250 and immunoblotted as previously described except that either a polyclonal anti-CYP2B (1:1000; Fitzgerald) or a polyclonal anti-CYP2E1 antibody (1:1000, Fitzgerald) was used for probing followed by a peroxidase-conjugated anti-rabbit IgG antibody (1:20000; Millipore Corporation, Billerica, MA) for detection. MCID Elite imaging software (Interfocus Imaging Ltd, Linton, UK) was used to measure band densities from the films.
Figure 26. Linear and specific detection of CYP2B6 and CYP2E1 protein.  
The amount of brain membrane protein loaded for immunoblotting was within the linear range of 
detection for CYP2B6 (A) and CYP2E1 (B) protein. Brain CYP2B6 and CYP2E1-immunoreactive 
protein bands co-migrated with human cDNA-expressed CYP2B6 and CYP2E1, 
respectively. No cross reactivity with cDNA-expressed human CYPs was observed for the anti-
CYP2B (C) and anti-CYP2E1 antibodies (D). CYP2B6 and CYP2E1 were loaded at 0.25 pmol, 
all other CYPs were loaded at 1 pmol, monkey liver was loaded at 5 μg.
RNA isolation, cDNA synthesis and mRNA quantification

RNA isolation, cDNA synthesis, PCR amplification and mRNA quantification were performed as previously described (Ferguson et al, 2011) with minor modifications. The mRNA sequence for African green monkey CYP2B6 and CYP2E1 were unavailable, therefore primer specificity was based on alignment between human and rhesus macaque (Macaca mulatta) CYP2B6 and CYP2E1 sequences; PCR products were subsequently sequenced (primer and PCR product sequences shown in supplementary Figure 33).

Statistics

Statistical methods are reported within the results section.

Results

Monkeys consistently self-administered ethanol throughout the study

The average daily ethanol consumption (from day 29 to 63) was 3.0 ± 0.6 g ethanol/kg, resulting in an average blood ethanol concentration of 26.1 ± 2.6 mM (0.12% by volume) as previously described (Ferguson et al., 2011). There was no significant difference in mean ethanol consumption between the ethanol-only and the ethanol + nicotine groups.

CYP2B6 protein was induced in monkey brain by ethanol self-administration and nicotine treatment

Assessment of CYP2B6 protein levels by immunocytochemistry

Ethanol self-administration increased CYP2B6 protein expression in the frontal cortex (layers II-VI), caudate, putamen, and cerebellum (granular, Purkinje cell and molecular layers) (Table 16,
Nicotine treatment also increased CYP2B6 protein in the frontal cortex (layer II-IV), hippocampus (CA2 region), putamen and cerebellum (Purkinje cell layer) (Table 16, Figure 27). In brain regions where ethanol and nicotine were both shown to independently increase CYP2B6 protein (i.e. layers II-IV of the frontal cortex), the ethanol + nicotine animals had greater CYP2B6 immunoreactivity compared to the ethanol-only and the nicotine-only animals, suggesting a greater effect of combined ethanol and nicotine exposure compared to either drug alone (Table 16, Figure 27).
# Table 16. Summary of brain CYP2B6 and CYP2E1 immunocytochemical staining

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>CYP2B6</th>
<th>CYP2E1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>E</td>
</tr>
<tr>
<td><strong>Frontal Cortex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Layer II-VI</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>White matter</td>
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<td>-</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentate gyrus (molecular layer)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Dentate gyrus (granular layer)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Dentate gyrus (polymorphic layer)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CA1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CA2</td>
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<td>+</td>
</tr>
<tr>
<td>CA3</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Caudate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Putamen</strong></td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Substantia nigra</strong></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Cerebellum</strong></td>
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<td>Molecular</td>
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<tr>
<td>Purkinje cells</td>
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<td>+++</td>
</tr>
<tr>
<td>Granular</td>
<td>+/-</td>
<td>++</td>
</tr>
<tr>
<td>White matter</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Key: control (C), ethanol-only (E), nicotine-only (N) and combined ethanol and nicotine (E +N), very strong (+++), strong (++), moderate (+), weak (+), and no staining (-)
Figure 27. Induction of CYP2B6 protein by ethanol self-administration and nicotine treatment in monkey brain measured by immunocytochemistry.

Cells in layers II-VI of the frontal cortex (FC) showed weak staining for CYP2B6 in the control animals (B), moderate staining in the ethanol-only (C) and nicotine-only animals (D), and strong staining in the ethanol + nicotine animals (E). In the caudate (CD), cellular staining for CYP2B6 was moderately intense in the control (G) and nicotine-only animals (I), and strong in the ethanol-only (H) and ethanol + nicotine animals (J). In the granular (g) and molecular (m) layers of the cerebellum (CB), CYP2B6 staining was very weak in the control animals (L) and nicotine-only animals (N), and moderately intense in the ethanol-only (M) and ethanol + nicotine animals (O). In the Purkinje cells (pc) of the cerebellum, CYP2B6 staining was weak in the control animals (L), moderately intense in the in the nicotine-only animals (N), strong in the ethanol-only animals (M) and very strong in the ethanol + nicotine animals (O). CYP2B6 immunoreactivity was barely detectable in the absence of primary antibody in the frontal cortex (A), caudate (F) and cerebellum (K). Scale bars =100 mm.
**Assessment of CYP2B6 protein levels by immunoblotting**

CYP2B6 protein expression was measured in seven brain regions by immunoblotting (Figure 28). In the control monkeys, CYP2B6 expression varied significantly among the brain regions that were assessed (one-way ANOVA, p<0.01) (Figure 28A). For some brain regions, the induced CYP2B6 protein levels were above the linear range of detection. To avoid protein underestimation due to saturation of the immunoblotting assay, the amount of brain membrane protein loaded onto the gels was reduced by the fold induction over control (e.g., inset Figure 28B). Once within the linear range of detection, the final protein levels were determined according to the relative densities and then corrected for the amount of protein loaded.
Figure 28. Region-specific induction of CYP2B6 protein by ethanol self-administration in monkey brain.

(A) CYP2B6 protein levels among brain regions in the control monkeys (n = 10 per region). (B) Fold-change in CYP2B6 protein levels for each brain region compared to the control group (n = 9-10 per group for each region). The dotted line represents CYP2B6 protein levels in the control group. *p<0.05, significantly different compared to same region in the control group; #p<0.05, significantly different compared to same region in the nicotine-only group; †p<0.05, significantly different compared to same region in the ethanol-only group. Frontal cortex (FC), hippocampus (HC), caudate (CD), putamen (PT), thalamus (TH), substantia nigra (SN), cerebellum (CB). A representative blot is shown to illustrate the basal and induced levels of CYP2B6 protein in monkey caudate. Lanes 1-4 were loaded with 40 μg of caudate membrane protein from control animals (C1-4). Lanes 5-8 were loaded with 40 μg of caudate membrane protein from animals in the ethanol-only group (E1-4). The same samples that were used in lanes 5-8 (E1-4) were diluted approximately 3.3-4.2-fold and loaded in lanes 9-12 (amount of membrane protein loaded: lane 9 = 9 μg, lane 10 = 12 μg, lane 11 = 10 μg and lane 23 = 11 μg). Coomassie blue staining is shown to indicate the amount of membrane protein loaded among lanes.
Multiple one-way ANOVAs were used to compare CYP2B6 protein levels between groups for each brain region separately. Significant differences were detected in the caudate (F[3, 35] = 14.47, p < 0.001), putamen (F[3, 36] = 6.92, p < 0.01), cerebellum (F[3, 36] = 3.81, p < 0.05), and thalamus (F[3, 36] = 8.01, p < 0.001). Bonferroni post-hoc testing indicated that the ethanol-only group had significantly higher CYP2B6 protein levels in the caudate (3.2-fold, p < 0.001), putamen (1.5-fold, p < 0.01) and cerebellum (1.6-fold, p < 0.05) compared to the control group, consistent with the results from the ICC analysis (Figure 28B, Table 16). Similarly, compared to the control group, the ethanol + nicotine group also had higher levels of CYP2B6 protein in the caudate (2.4-fold, p < 0.01), putamen (1.4-fold, p < 0.05) and cerebellum (1.6-fold, p < 0.05), which was also observed with ICC (Figure 28B, Table 16). In addition, the ethanol-only and ethanol + nicotine groups had significantly higher levels of CYP2B6 (p < 0.05) compared to the nicotine-only group in the caudate, putamen and cerebellum, which was again consistent with the results from the ICC analysis (Figure 28B, Table 16).

In the thalamus, CYP2B6 levels in the ethanol + nicotine group were higher compared to the control (1.5-fold, p < 0.01), ethanol-only (1.3-fold, p < 0.01) and nicotine-only groups (1.7-fold, p < 0.001), demonstrating an interactive effect between ethanol and nicotine. There were no significant differences in CYP2B6 protein expression between study groups in the frontal cortex (F[3, 36] = 2.86, p = 0.05), hippocampus (F[3, 36] = 2.29, p = 0.10) or substantia nigra (F[3, 36] = 1.63, p = 0.20) (Figure 28B). The regional pattern of CYP2B6 expression detected in the brains of monkeys exposed to ethanol and/or nicotine closely paralleled the expression of CYP2B6 protein previously observed in brains from human alcoholics and smokers (Miksys et al., 2003) (Figure 29, Table 17).
Figure 29. Alcoholic smokers have higher levels of CYP2B6 in specific brain regions.

(A) Fold-change in CYP2B6 protein levels in human alcoholics (n = 9) for each brain region compared to non-alcoholics (n = 8; PT, n= 5; HC and CB, n = 17). The dotted line represents CYP2B6 protein levels in the non-alcoholics. (B) Fold-change in CYP2B6 protein levels in human smokers (n = 12; PT, n=10) for each brain region compared to non-smokers (n = 7; PT, n = 4; HC and CB n = 17). The dotted line represents CYP2E1 protein levels in the non-smokers. *p<0.05, significantly different compared to same region in control individuals, either non-alcoholic (A) or non-smokers (B). Frontal cortex (FC), hippocampus (HC), caudate (CD), putamen (PT), thalamus (TH), substantia nigra (SN), cerebellum (CB). This figure was generated using previous published data (Miksys et al, 2003).
Table 17. Demographic information for human tissue donors

<table>
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<th>Sample</th>
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<th>Alcoholism</th>
<th>Smoking</th>
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<td>Chronic</td>
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</tbody>
</table>

*Human brain tissues were obtained from the University of Miami Brain Endowment Bank and were acquired during routine autopsy.

†Code used in Miksys et al, 2003

‡Postmortem interval (PMI)
CYP2E1 protein was induced in monkey brain by ethanol self-administration and nicotine treatment

Assessment of CYP2E1 protein levels by immunocytochemistry

Ethanol self-administration induced CYP2E1 protein expression in the hippocampus (granular layer of the dentate gyrus and CA2 region) and the cerebellum (granular, Purkinje cell and molecular layers) (Table 16, Figure 30). Nicotine treatment also increased CYP2E1 protein in the frontal cortex (layers II-VI), putamen and cerebellum (granular, Purkinje cell and molecular layers) (Table 16, Figure 30). In the cerebellum, where ethanol and nicotine both independently increased CYP2E1 protein, the ethanol + nicotine animals had greater CYP2E1 immunoreactivity compared to the ethanol-only and the nicotine-only animals, suggesting a greater effect of combined ethanol and nicotine exposure compared to either drug alone (Table 16, Figure 30).
Figure 30. Induction of CYP2E1 protein by ethanol self-administration and nicotine treatment in monkey brain measured by immunocytochemistry.

In the granular layer (g) of the dentate gyrus in the hippocampus (HC), staining for CYP2E1 was weak in the control (B) and nicotine-only animals (D), and moderately intense in the ethanol-only (C) and ethanol + nicotine animals (E). Staining for CYP2E1 was weak in the polymorphic (p) and molecular layers (m) of the dentate gyrus for all groups (B-E). In the granular (g) and molecular (m) layers of the cerebellum (CB), CYP2E1 staining was weak in the control animals (G), moderately intense in the ethanol-only (H) and nicotine-only animals (I), and strong in the ethanol + nicotine animals (J). In the Purkinje cells (pc) of the cerebellum, CYP2E1 staining was moderately intense in the control animals (G), strong in the ethanol-only (H) and nicotine-only animals (I), and very strong in the ethanol + nicotine animals (J). In the putamen (PT), cellular staining for CYP2E1 was weak in the control (L) and ethanol-only animals (M), and strong in the nicotine-only (N) and nicotine + ethanol animals (O). CYP2E1 immunoreactivity was barely detectable in the absence of primary antibody in the dentate gyrus of the hippocampus (A), cerebellum (F) and putamen (K). Scale bars =100 μm.
Assessment of CYP2E1 protein levels by immunoblotting

CYP2E1 protein expression was measured in seven brain regions by immunoblotting (Figure 31). In the control monkeys, CYP2E1 varied significantly among the brain regions (one-way ANOVA, p<0.05) (Figure 31A). For some brain regions, the induced CYPE1 protein levels were above the linear range of detection (e.g., inset Figure 31B). To avoid protein underestimation due to saturation of the immunoblotting, the amount of brain membrane protein loaded onto the gels was diluted and then corrected for the amount of protein loaded.

Multiple one-way ANOVAs were used to compare CYP2E1 protein levels between groups for each brain region separately. A significant difference was detected in the frontal cortex (F[3,34] = 3.634, p < 0.05) and the putamen (F[3,34] = 6.484, p < 0.01). Bonferroni post-hoc testing indicated that the nicotine-only group had significantly higher CYP2E1 protein levels in the frontal cortex (1.6-fold, p < 0.05) and putamen (2.0-fold, p < 0.05) compared to the control group, consistent with the results from the ICC analysis (Figure 31B, Table 16). Likewise, compared to the control group, the ethanol + nicotine group also had higher levels of CYP2E1 protein in the frontal cortex (1.5-fold, p < 0.05) and putamen (1.8-fold, p < 0.01), which was also observed by ICC (Figure 31B, Table 16). In addition, the nicotine-only and ethanol+nicotine groups had significantly higher levels of CYP2E1 (p < 0.05) compared to the ethanol-only group in the frontal cortex and putamen, which was again consistent with the results from the ICC analysis (Figure 31B, Table 16).
Figure 31. Region-specific induction of CYP2E1 protein by ethanol self-administration in monkey brain.

(A) CYP2E1 protein levels among brain regions in the control monkeys (n = 10 per region). (B) Fold-change in CYP2E1 protein levels for each brain region compared to the control group (n = 9-10 per group for each region). The dotted line represents CYP2E1 protein levels in the control group. *p<0.05, significantly different compared to same region in the control group; †p<0.05, significantly different from same region in the ethanol-only group. A representative blot is shown to illustrate the basal and induced levels of CYP2E1 protein in monkey putamen. Lanes 1-4 were loaded with 30 μg of putamen membrane protein from control animals (C1-4). Lanes 5-8 were loaded with 30 μg of putamen membrane protein from animals in the nicotine-only group (N1-4). The same samples that were used in lanes 5-8 (N1-4) were diluted approximately 2.5 to 3.3-fold and loaded in lanes 9-12 (amount of membrane protein loaded: lane 9 = 12 μg, lane 10 = 9 μg, lane 11 = 15 μg and lane 12 = 12 μg). Coomassie blue staining is shown to indicate the amount of membrane protein loaded among lanes.
There were no significant differences in CYP2E1 protein expression between study groups in the hippocampus (F[3, 36] = 1.75, p = 0.18), caudate (F[3,36] = 2.51, p = 0.74), substantia nigra (F[3, 36] = 0.26, p = 0.85), and cerebellum (F[3,36] = 1.05, p < 0.38). However, in the cerebellum, a noticeable but non-significant effect of nicotine was observed as shown by the higher levels of CYP2E1 protein in the nicotine-only and nicotine + ethanol groups compared to the control and ethanol-only groups. This increase in CYP2E1 protein levels in the cerebellum by nicotine is consistent with our ICC assessment (Figure 31B, Table 16). The pattern of CYP2E1 expression detected in the brains of monkeys exposed to ethanol and/or nicotine closely paralleled the expression of CYP2E1 protein observed in brains from human alcoholics and smokers (Figure 32, Table 17).
Figure 32. Human smokers have elevated levels of CYP2E1 protein in specific brain regions

(A) Relative CYP2E1 protein levels in alcoholics (n = 9) compared to non-alcoholics (FC and CD, n = 10; PT, n = 5; SN, n=7, HC and CB, n = 18) among brain regions. (B) Relative CYP2E1 protein levels in smokers (FC, HC and CD n = 12; PT, SN and CB, n=10) compared to non-smokers (FC and CD, n = 7; PT, n = 4; SN, n = 6, HC and CB n = 17) among brain regions. Excluding female cases did not alter the results of the statistical analyses. There was no significant correlation (Pearson correlation, p > 0.05) between post mortem interval and the levels of CYP2E1 in any of the brain regions assessed. There was no significant difference (Student’s t-test, p > 0.05) in age or post mortem interval between alcoholics and non-alcoholics. *p<0.05, significantly different compared to the same brain region in non-smokers. FC, Frontal cortex; HC, hippocampus; CD, caudate; PT, putamen; SN, substantia nigra; CB, cerebellum.
CYP2B6 and CYP2E1 mRNA was not significantly altered by ethanol self-administration or nicotine treatment in monkey brain

CYP2E1 and CYP2B6 mRNA levels in the putamen and frontal cortex were assessed by quantitative RT-PCR to investigate whether transcriptional activation and/or increased mRNA stability contributed to ethanol-mediated induction of brain CYP2B6 protein or nicotine-mediated induction of brain CYP2E1 protein. CYP2B6 mRNA levels were not significantly different between treatment groups in the putamen (F[3, 24] = 2.38, p = 0.10), although substantial induction of CYP2B6 protein by ethanol was observed in this region. Likewise, CYP2E1 mRNA levels were not significantly different between treatment groups in the putamen (ANOVA, F[3, 26] = 0.03, p = 0.99), despite an induction of CYP2E1 protein by nicotine in this region. In the frontal cortex, the treatment groups did not differ in the levels of CYP2B6 mRNA (F[3, 25] = 0.30 p = 0.83) or CYP2E1 mRNA (F[3, 26] = 0.32, p = 0.81). The AGM CYP2B6 and CYP2E1 PCR fragments had high sequence homology to cDNA derived from human CYP2B6 and CYP2E1 mRNA (Figure 33).

African green monkey CYP2B6 and CYP2E1 PCR amplified cDNA fragments were sequenced and found to be homologous with both human and rhesus macaque (Macaca mulatta) CYP2B6 and CYP2E1 mRNA (Figure 33). The AGM CYP2B6 PCR fragment had 99.6% sequence homology with rhesus macaque (Macaca mulatta) CYP2B6 mRNA (1 out of 242 nucleotides were different), and 93.8% sequence homology with human CYP2B6 mRNA (15 out of 242 nucleotides were different). The AGM CYP2E1 PCR fragment had 100.0% sequence homology with rhesus macaque CYP2E1 mRNA (all 114 nucleotides were identical), and 94.7% homology with human CYP2E1 mRNA (6 out of 114 nucleotides were different).
Figure 33. African green monkey CYP2B6 and CYP2E1 PCR amplified cDNA fragments were sequenced and found to be homologous with human and macaque (Macaca mulatta) CYP2B6 (A) and CYP2E1 mRNA (B), respectively.

The primer sequences used to amplify AGM cDNA fragments are underlined (forward primers solid line, reverse primers dotted line) and differences among the AGM, human and macaque sequences are highlighted. Nucleotides are numbered consecutively from the A of the ATG start codon. 2B6hum: human CYP2B6; 2B6mac: macaque CYP2B6; 2B6agm: African green monkey CYP2B6; 2E1 hum: human CYP2E1; 2E1mac: macaque CYP2E1; 2E1agm: African green monkey CYP2E1
Discussion

Brain CYPs are active in situ and can meaningfully contribute to the local metabolism of centrally acting drugs and toxins (Miksys and Tyndale, 2009; Khokhar and Tyndale, 2011; 2012). In humans, interindividual variation in brain CYP activity due to age, genetic variation or exposure to inducers and inhibitors may contribute to observed differences in response to psychoactive drugs and susceptibility to neurotoxicity. In this study we identified ethanol and nicotine as inducers of CYP2B6 and CYP2E1 in the primate brain. Given the large proportion of the population that is exposed to ethanol and nicotine, these findings have important implications.

In rats, CYP2B and CYP2E1 can be induced in certain brain regions by ethanol and/or nicotine treatment (Miksys et al., 2000; Howard et al., 2003); however, the regional pattern of CYP2B1 and CYP2E1 induction by ethanol and/or nicotine in rat brain does not coincide with the specific regions where CYP2B6 and CYP2E1 protein levels are elevated in human alcoholics and smokers (Howard et al., 2003; Miksys et al., 2003). Rats are not the best translational model to study the regional and cellular pattern of CYP induction in the human brain, due to important differences in neuroanatomy and brain CYP expression pattern between rats and humans. For example, CYP2B is highly expressed and inducible by nicotine in the rat olfactory bulb and olfactory tubercle (Miksys et al., 2000); however, these regions are proportionally much smaller (compared to brain size) in primates (Smith and Bhatnagar, 2004). In contrast, the AGM is an established model of human CYP expression and regulation in the brain (Joshi and Tyndale, 2006b; Lee et al., 2006b; Mann et al., 2008). The demonstration of CYP2B6 and CYP2E1 induction in monkey brain strongly suggests that this induction also occurs in humans.
Monkeys consistently self-administered ethanol throughout the study at levels comparable to moderate human consumption (Ferguson et al., 2011). The monkeys were identified as moderate consumers based on their blood ethanol concentration after the alcohol access period (average = 26.1 ± 2.6 mM or 0.1% by volume). The use of blood ethanol concentration, and not simply ethanol consumption, takes into consideration the 2-fold greater ethanol elimination rate in AGM compared to humans (Ervin et al., 1990). The total daily dose of nicotine administered to the monkeys (0.5 mg/kg bid) was similar to the average amount of nicotine acquired by smokers (0.2-1.1 mg/kg) (Benowitz and Jacob, 1984). This dosing regimen produced mean nicotine plasma levels of approximately 25 ng/ml for 16 h/day (Lee et al, 2006), which is within the range of nicotine plasma levels achieved by smokers during waking hours (10-50 ng/ml) (Benowitz NL, 1990). Therefore, nicotine-treated monkeys were similar to human smokers with respect to the level and daily duration of nicotine in plasma, but differed in the pattern of nicotine intake (two injections per day compared to multiple cigarettes per day).

Our results demonstrate the utility of using both ICC and immunoblotting to assess protein expression in tissues. ICC revealed cellular induction of CYP2B6 and CYP2E1 by ethanol and nicotine in monkey brain, while immunoblotting, which does not always detect differences in protein expression among specific cell populations, was used to quantify changes in the overall levels of CYP2B6 and CYP2E1 within brain regions. The pattern of CYP2B6 and CYP2E1 expression detected in the brains of monkeys exposed to ethanol and/or nicotine closely paralleled the expression of CYP2B6 and CYP2E1 protein observed in the brains from human alcoholics and smokers (Howard et al., 2003; Miksys et al., 2003). For example, the ethanol-only monkeys and human alcoholics both had elevated levels of CYP2B6 protein in the putamen and elevated levels of CYP2E1 protein in the dentate gyrus of the hippocampus, but unaltered
expression of either enzyme in the substantia nigra (Howard et al., 2003; Miksys et al., 2003). The nicotine-only monkeys and human smokers both had elevated levels CYP2B6 protein in the CA2 region of the hippocampus and elevated levels of CYP2E1 protein in the putamen, but unaltered expression of either enzyme in the caudate (Howard et al., 2003; Miksys et al., 2003). Based on these similarities, our results strongly suggest that ethanol and nicotine are responsible, at least in part, for the higher levels of CYP2B6 and CYP2E1 in the brains of human alcoholics and smokers. Our findings are consistent with the results of a previous monkey study which also demonstrated the induction of CYP2B6 and CYP2E1 in monkey brain after chronic nicotine treatment (Joshi and Tyndale, 2006b; Lee et al., 2006b). This is the first study to report the induction CYP2B6 and CYP2E1 by ethanol self-administration, either alone or in combination with nicotine, in the primate brain.

Approximately 70-75% of alcoholics are heavy smokers (Bobo and Husten, 2000) and 90% of smokers regularly consume alcohol (Shiffman and Balabanis, 1995), which makes it a challenge to study the independent effects of alcohol consumption and tobacco use in human autopsy brain samples. Given the frequent co-use and co-abuse of alcohol and tobacco, it was important to assess the combined effect of ethanol and nicotine on brain levels of CYP2B6 and CYP2E1 in the monkeys. In certain brain regions (i.e. frontal cortex, putamen and cerebellum), combined ethanol and nicotine exposure resulted in a greater induction of CYP2B6 or CYP2E1 than either drug alone, suggesting additive or even synergistic effects. The induction of CYP2E1 and CYP2B6 in the brain can result in altered susceptibility to neurotoxins, metabolic tolerance and changes in the efficacy of centrally acting drugs. Our results imply that the potential effects of elevated CYP2B6 and CYP2E1 in the brain may be exacerbated in individuals who both
regularly consume alcohol and smoke cigarettes (or those who are taking nicotine therapeutically).

CYP2B6 and CYP2E1 mRNA levels in the putamen and frontal cortex were not affected by ethanol self-administration or nicotine treatment, suggesting that the induction of CYP2B6 protein by ethanol and CYP2E1 protein by nicotine in these regions do not occur through an increase in transcription or mRNA stability. This is consistent with a previous study showing that nicotine induces CYP2E1 protein, but not CYP2E1 mRNA, in multiple regions of rat brain (Joshi and Tyndale, 2006a). Our study is the first to investigate the effect of ethanol on CYP2B6 mRNA levels in the brain. In monkeys, hepatic CYP2B6 protein levels were induced after ethanol self-administration without a corresponding increase in CYP2B6 mRNA levels (Ferguson et al., 2012), suggesting a similar non-transcriptional mechanism for the induction of CYP2B6 by ethanol in the brain and liver.

Studies in rodents have demonstrated that selectively manipulating the levels and activity of CYPs in the brain (while leaving peripheral CYPs unaffected) can have a significant impact on the response to centrally acting compounds. For example, rats were given intracerebroventricular injections of a CYP2B inhibitor that selectively inhibited the enzyme in the brain but not the liver. Compared to vehicle-treated animals, the inhibitor-treated animals showed increased sleep time upon systemic administration of the anesthetic propofol, which is metabolically inactivated by CYP2B, and decreased neurotoxicity when systemically exposed to the pesticide chlorpyrifos, which is metabolically activated by CYP2B (Khokhar and Tyndale, 2011; 2012). Pharmacokinetic analysis demonstrated that the altered response to propofol and chlorpyrifos observed in the inhibitor-treated animals was due to decreased brain CYP2B metabolism and altered metabolite levels in the brain (Khokhar and Tyndale, 2011; 2012).
Collectively, these studies demonstrate that alterations in CYP-mediated metabolism in the brain can have a meaningful impact on drug response and risk for neurotoxicity.

Given the variety of centrally acting drugs, toxins and endogenous neurochemicals that are metabolized by CYP2B6 and CYP2E1, elevated levels of these enzymes in the brain could potentially have wide-ranging and important consequences. Conducting human studies to demonstrate the association between elevated brain CYP levels and altered response to centrally acting compounds is a challenge. However, it is widely speculated that elevated levels of brain CYPs, including CYP2B6 and CYP2E1, contribute to altered drug response, neurotoxicity and central nervous system diseases (Ferguson and Tyndale, 2011). For example, elevated brain levels of CYP2B6 have been associated with increased risk for neurotoxicity from compounds such as chlorpyrifos and 9-methoxy-N²-methylellipticinium acetate (MMEA) (Upadhya et al., 2002; Khokhar and Tyndale, 2012), and interindividual variation in brain CYP2B6 may contribute to differences in cigarette craving and ability to quit smoking (Lerman et al., 2002). Elevated levels of brain CYP2E1 have been implicated in the pathophysiology of chronic solvent-induced encephalopathy (Kezic et al., 2006), Parkinson’s disease (Kaut et al., 2012) and alcohol-induced neurodegeneration (Zhong et al., 2012).

In conclusion, we demonstrated the region-specific induction of CYP2B6 and CYP2E1 by ethanol self-administration and nicotine treatment in monkey brain. We also show that, in certain brain regions, combined ethanol and nicotine exposure resulted in greater induction of CYP2B6 or CYP2E1 than either drug alone. The induction of CYP2B6 and CYP2E1 in the human brain by ethanol and nicotine could contribute to observed interindividual variation in the response to centrally acting drugs and toxins.
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Significance of chapter

In this study, we showed that ethanol and nicotine, both alone and in combination, can induce brain CYP2E1 and CYP2B6 in a region- and cell-specific manner. Our results strongly suggest that ethanol and nicotine are responsible, at least in part, for the elevated levels of CYP2E1 and CYP2B6 in human alcoholics and smokers. Our immunohistochemistry data suggest that in some cell-populations combined ethanol and nicotine exposure may result in a greater induction of CYP2E1 and/or CYP2B6 than either drug alone. Recently, several studies have demonstrated that CYPs in the brain are functional and alterations in the expression of brain CYPs can have a meaningful impact on the response to various drugs and toxins (Ferguson and Tyndale, 2011; Khokhar and Tyndale, 2011). Therefore the induction of brain CYP2E1 and CYP2B6 could potentially have important clinical consequences, such as altered efficacy of centrally acting drugs and changes in susceptibility to neurotoxins.
Section 3: General discussion

1. Effects of ethanol and nicotine on hepatic CYPs

The liver is quantitatively the most important site for CYP-mediated metabolism of xenobiotics. The induction or downregulation of hepatic CYPs can affect the bioavailability and/or clearance of drugs, leading to altered drug efficacy. Changes in the activity of hepatic CYPs can also affect the bioactivation and detoxification of toxic compounds, leading to altered susceptibility to toxicity and disease. In Chapters 1 and 2, we demonstrated that ethanol and/or nicotine can alter the expression and activity of hepatic CYP2E1, CYP2B6 and CYP2A6 in monkeys. In the following section, the regulation of these three hepatic CYPs by ethanol and/or nicotine will be discussed. In addition, some key examples demonstrating the potential consequences of altered CYP2E1, CYP2B6 and CYP2A6 activity in the liver will be presented.

1.1 Hepatic CYP2E1

1.1.1 Induction of hepatic CYP2E1 by ethanol and nicotine

In humans, moderate ethanol consumption is associated with faster in vivo CZN clearance, which has been attributed to the induction of hepatic CYP2E1 protein and activity by ethanol (Howard et al., 2001; Oneta et al., 2002). In Chapter 1, we demonstrated that moderate ethanol consumption induces hepatic CYP2E1 protein and activity in primates, leading to decreased CZN AUC after oral administration.

The induction of CYP2E1 protein by ethanol in AGM liver occurred via a post-transcriptional mechanism (Chapter 1). This is consistent with a number of studies showing that ethanol consumption can induce hepatic CYP2E1 by protein stabilization (Roberts et al.,
Ethanol is known to inhibit the proteasomal degradation of hepatic CYP2E1, increasing its half-life from approximately 7 to 38 hours in rats (Roberts et al., 1995; Zanelli et al., 2000). The precise molecular events responsible for the ethanol-mediated inhibition of CYP2E1 proteasomal degradation are not fully understood, but a variety of mechanisms have been proposed. For example, the binding of ethanol to CYP2E1 may shield the enzyme from post-transcriptional modifications (e.g. ubiquitination and phosphorylation) or interactions with chaperone proteins (e.g. heat shock protein 90) that mediate proteasomal degradation (Banerjee et al., 2000; Morishima et al., 2005; Kitam and Maksymchuk, 2012).

Although not observed in our study, hepatic CYP2E1 mRNA levels can also be induced by chronic ethanol treatment (Ronis et al., 1993). The induction of hepatic CYP2E1 mRNA occurs at high plasma ethanol concentrations (> 65 mM, 0.3% ethanol by volume) well above the levels achieved by ethanol self-administration in our monkey study (Ronis et al., 1993).

In Chapter 1, we showed that CYP2E1 activity positively correlates with average daily ethanol intake. A similar dose dependent effect of ethanol on CYP2E1 protein levels has been demonstrated in rats (Howard et al., 2001). The magnitude of CYP2E1 induction by ethanol is also dependent on the duration of ethanol exposure. For example, in humans, moderate ethanol consumption (40 g/day) for a period of 28 days resulted in a progressive increase in CZN clearance, such that CZN clearance was significantly faster on day 28 compared to day 21 and after ethanol withdrawal CYP2E1 levels returned to baseline within 3 days (Oneta et al., 2002). Given the large variation in the amount and frequency of alcohol consumption among individuals, future studies should determine how different patterns of alcohol use affect CYP2E1 activity. For example, intermittent binge drinking is a pattern of alcohol consumption that is becoming increasingly prevalent, especially among young adults (Centers for Disease Control
and Prevention, 2012). Studies should assess how this pattern of high dose, non-daily ethanol consumption affects CYP2E1 levels. Also, many health and wellness guidelines suggest drinking 1-2 glasses of wine daily (Panagiotakos et al., 2003). Studies should assess how consuming low doses of ethanol daily over a period of several months affects CYP2E1 activity. Cigarette smoking can enhance in vivo CZN metabolism in humans, suggesting that one or more chemicals found in cigarette smoke can induce CYP2E1 activity (Benowitz et al., 2003). In Chapter 1 we showed that chronic nicotine treatment can induce hepatic CYP2E1 protein levels and activity in AGM, resulting in a decreased CZN C\textsubscript{max} and AUC. Our results suggest that nicotine exposure causes, or at least contributes to, the enhanced CYP2E1 activity and altered xenobiotic metabolism in smokers.

Nicotine treatment induced CYP2E1 in AGM liver via a post-transcriptional mechanism (Chapter 2). This is consistent with a previous study showing that nicotine can increase CYP2E1 protein but not mRNA in rat liver (Howard et al., 2001; 2003). It is unlikely that the induction of CYP2E1 by nicotine occurs via protein stabilization. Nicotine does not competitively inhibit CYP2E1-hydroxylation of CZN, suggesting that nicotine does not interact directly with the active site of CYP2E1 to stabilize the enzyme (Howard et al., 2001). Also, using an established in vitro method for assessing CYP2E1 stability (Huan and Koop, 1999), nicotine treatment did not affect the rate of CYP2E1 degradation in two different cell lines (Micu et al., 2003). A potential mechanism for the induction of CYP2E1 by nicotine may be via translational activation. Pyridine is a compound that is structurally similar to nicotine and it can increase CYP2E1 protein synthesis, in the absence of transcriptional activation, by enhancing the rate of CYP2E1 translation (Kim et al., 1990; Kim and Novak, 1990). The effect of nicotine on CYP2E1 translational efficiency could be assessed by performing polysome profiling.
experiments (Kuersten et al., 2013).

The total daily dose of nicotine administered to the monkeys (0.5 mg/kg bid) was similar to the average amount of nicotine acquired by a human smoker (0.2–1.1 mg/kg/day) (Benowitz and Jacob, 1984). This dosing regimen produced mean nicotine plasma levels of approximately 25 ng/ml for 16 h/day (Lee et al., 2006b), which is within the range of nicotine plasma levels achieved by an average smoker during waking hours (10-50 ng/ml) (Benowitz NL, 1990). In monkeys and rats, chronic treatment with lower doses of nicotine (ranging from 0.005 – 0.6 mg/kg daily) can also induce hepatic CYP2E1 in a dose dependent manner (Howard et al., 2001; Micu et al., 2003; Lee et al., 2006c), suggesting that hepatic CYP2E1 activity may be induced in light smokers, but to a lesser extent compared to heavy smokers. A time course experiment in rats revealed that seven, but not five, days of nicotine treatment (at a daily dose of 1.0 mg/kg) was necessary to induce CYP2E1, and after nicotine treatment was stopped CYP2E1 levels returned to baseline within 24 hrs (Micu et al., 2003). Therefore, hepatic CYP2E1 activity may not be induced in non-daily smokers.

Our results suggest that nicotine is responsible, at least in part, for the higher enhanced CZN clearance that is observed in human smokers (Benowitz et al., 2003). Interestingly, the administration of a 21-mg transdermal nicotine patch twice daily (equivalent to 0.3 mg nicotine/kg bid for a 70 kg human) for 10 days did not affect CZN clearance in humans (Hukkanen et al., 2009). This is in contrast to our results and the previous studies in monkeys and rats demonstrating that nicotine delivered by subcutaneous injections can induce CYP2E1 protein levels and activity (Howard et al., 2001; Lee et al., 2006c). Currently it is unclear why nicotine injected subcutaneously (0.3 or 0.5 mg/kg bid) induced CYP2E1 in AGM, but a similar dose of nicotine administered via transdermal patch (approximately 0.3 mg/kg bid) did not
induce CZN clearance in humans. A possible explanation could be that the induction of CYP2E1 by nicotine is a response that occurs in non-human primates, but not in humans. This explanation is not likely. Monkey and human CYP2E1 protein are highly homologous and show similar regulation by a variety of inducers (See section 8.3.1 of introduction); the demonstration of CYP2E1 induction by nicotine in AGM strongly suggest that nicotine is also an inducer of CYP2E1 in humans. Another explanation could be that the peak nicotine plasma levels achieved from a transdermal nicotine patch are not sufficient to induce CYP2E1 and alter CZN clearance. Our nicotine treatment paradigm (0.5 mg/kg nicotine s.c., bid) was estimated to produce average nicotine plasma levels of approximately 25 ng/ml with a $C_{\text{max}}$ of approximately 250 ng/ml in AGM, whereas a 21-mg transdermal nicotine patch, which gradually releases nicotine into the blood stream, produces average nicotine plasma levels of approximately 20 ng/ml with a $C_{\text{max}}$ of 20-30 ng/ml in humans (Gupta et al., 1995). Further studies are needed to assess the impact of nicotine route of administration on the induction of hepatic CYP2E1.

Combined ethanol self-administration and nicotine treatment had an additive effect on the induction of hepatic CYP2E1 protein levels and in vitro activity. In the context of our study, “additive effect” indicates that ethanol and nicotine in combination produce a total effect that is equal to the sum of each drug’s individual effect. However, “additive effect” has multiple definitions, including statistical definitions that may not accurately describe our data (Chou, 2010). Our results suggest that individuals who are exposed to ethanol and nicotine in combination may experience greater changes in CYP2E1-mediated xenobiotic metabolism and disposition compared to those who are exposed to either drug alone.
1.1.2 Clinical consequences of elevated hepatic CYP2E1

*Example 1 – Increased risk for chemically-mediated cancer.* From a toxicological perspective, CYP2E1 is a very important enzyme. It metabolically activates several drugs (i.e. acetaminophen, halothane) and numerous industrial chemicals (e.g., vinyl chloride, n-hexane and trichloroethylene) to hepatotoxic and carcinogenic metabolites (Trafalis et al., 2010). Therefore, induction of hepatic CYP2E1 by ethanol and/or nicotine is a concern because it may cause increased susceptibility to toxicity from a variety of compounds. For example, vinyl chloride is primarily metabolized in the liver by CYP2E1 to chloroethylene oxide (Guengerich et al., 1991; Ghissassi et al., 1998) which is a genotoxic metabolite implicated in the development of liver cirrhosis, hepatic angiosarcoma and hepatocellular cancer in humans (Ward et al., 2001; Mastrangelo et al., 2004; Wang et al., 2011b). Among workers exposed to vinyl chloride, individuals homozygous for the high activity CYP2E1*5B gene variant had elevated markers of genotoxicity and chromosomal damage (Wang et al., 2010c). Also, workers exposed to vinyl chloride who regularly consumed alcohol and/or smoked cigarettes had significantly elevated markers of genotoxicity and chromosomal damage compared to workers exposed to vinyl chloride who did not drink alcohol or smoke, as well as individuals who drank alcohol and/or smoked but were not exposed to vinyl chloride (Ji et al., 2010; Kumar et al., 2013). Furthermore, alcohol consumption (>70 g/day) and vinyl chloride exposure were shown to act synergistically to increased risk for hepatocellular cancer, meaning that the risk associated with combined vinyl chloride and alcohol consumption was significantly greater than the sum of their independent risks (Mastrangelo et al., 2004). Collectively, these findings suggest that elevated CYP2E1 activity can increase the genotoxic effects of vinyl chloride and that alcohol consumption and/or smoking can potentiate vinyl chloride toxicity, possibly via the induction of
CYP2E1. There are more than 85 other xenobiotics that are bioactivated by CYP2E1 to produce toxic or carcinogenic metabolites (Trafalis et al., 2010); the induction of CYP2E1 by ethanol and/or nicotine can potentially increase the harmful effects of these compounds as well. These studies demonstrate the need to characterize the pattern of drinking and smoking (e.g. dose and frequency) that is required to induce hepatic CYP2E1 levels so we can identify individuals who may be at higher risk for toxicity from compounds that are than are metabolically activated by CYP2E1. Also, when setting occupational exposure limits for hazardous industrial chemicals, toxicologists should consider the potential induction of hepatic CYP2E1 by ethanol and/or nicotine and the possibility of heightened susceptibility to toxicity in alcohol consumers and smokers.

**Example 2 – Increased ROS production and risk for liver damage.** An important feature of CYP2E1 is its ability to produces ROS, such as superoxide anion radicals and hydrogen peroxide (Caro and Cederbaum, 2004). CYP2E1 can generate ROS in the absence of a substrate; however, the extent of ROS production by CYP2E1 is increased when a substrate is present (Caro and Cederbaum, 2004). Low levels of ROS are necessary for normal cellular function, but high levels of ROS can cause oxidative stress, which is characterized by lipid peroxidation, protein denaturation, DNA damage, and cell death (Caro and Cederbaum, 2004). Oxidative stress has been implicated in many different pathological conditions, such as alcoholic liver disease, cardiovascular diseases (atherosclerosis, reperfusion injury), cancer (e.g. hepatocellular cancer), diabetes and neurodegenerative diseases (e.g. alcohol-induced neurodegeneration, Alzheimer’s disease) (Kehrer, 1993; Knight, 1998). The induction of CYP2E1 in the liver and
extrahepatic tissues can potentially enhance ROS production and increase susceptibility to these ROS-related diseases.

Increased oxidative stress via CYP2E1 induction may have an important role in the mechanism by which ethanol causes liver injury and increases the risk for liver disease and hepatocellular cancer (Kuper et al., 2000; Caro and Cederbaum, 2004; Shih et al., 2012). In rodents, the severity of ethanol-induced liver pathology has been correlated with hepatic CYP2E1 and ROS levels (Nanji et al., 1994b; Morimoto et al., 1995). The induction of CYP2E1 by ethanol is mainly localized to the perivenular zone of the liver, precisely where ethanol-induced liver damage is maximal (Lieber, 1999). Humanized CYP2E1 knock-in mice treated chronically with ethanol exhibit liver damage (e.g. steatosis, necrosis) and oxidative stress; this response was not found in similarly treated Cyp2el-null mice (Lu et al., 2010). Collectively, these findings suggest a role for CYP2E1 induction in ethanol-mediated liver damage. Cigarette smoking is also a risk factor for developing hepatocellular cancer and negatively affects hepatocellular cancer survival rates (Kuper et al., 2000; Shih et al., 2012), which may be related to nicotine’s induction of CYP2E1 and subsequent ROS production. Furthermore, heavy ethanol consumption and smoking have an additive effect on the risk hepatocellular cancer, possibly due to the additive effect of ethanol and nicotine on CYP2E1 induction (Kuper et al., 2000; Shih et al., 2012).

CYP2E1 inhibitors could potentially be used as a treatment for alcoholic liver disease and to prevent alcohol-induced hepatocarcinogenesis. This would require the development of selective CYP2E1 inhibitors that can be administered to humans with minimal toxicity and side effects. S-allylmercaptocysteine is a selective CYP2E1 inhibitor derived from garlic that may meet this criteria (Sumioka et al., 2001; Xiao et al., 2013).
1.2. Hepatic CYP2B6

1.2.1 Induction of hepatic CYP2B6 by ethanol

Using tissue from a human liver bank, self-reported alcohol consumption (>14 drinks/week) was associated with higher hepatic CYP2B6 protein content and in vitro activity (Hesse et al., 2004). Consistent with these findings, we demonstrated that ethanol self-administration can increase hepatic CYP2B6 protein levels and in vitro activity (Chapter 2). We did not assess the impact of ethanol on in vivo CYP2B6 metabolism, but this can be tested in the future by administering bupropion to animals and measuring the formation of 6-hydroxybupropion in vivo (Kirchheiner et al., 2003).

In AGM, ethanol-mediated induction of hepatic CYP2B6 protein occurred without an increase in CYP2B6 mRNA (Chapter 2). This is consistent with another study that has demonstrated post-transcriptional regulation of CYP2B6 by ethanol in rats (Nanji et al., 1994a). A potential mechanism for the induction of CYP2B6 protein by ethanol is via decreased proteasomal degradation. In vitro studies show that CYP2B can be degraded by a proteasome-dependent process (Huan et al., 2004; Lee et al., 2008), and although ethanol is not a substrate for CYP2B6, it may bind to the enzyme at a peripheral site and inhibit post-translational modifications that are necessary for targeting the enzyme for degradation. A crystal structure for rabbit CYP2B4 showed a high affinity site on the surface of the enzyme that binds cyclohexylpentyl-β-D-maltoside, a solubilizing agent used in crystallization screens (Zhao et al., 2006), demonstrating that a CYP2B enzyme can bind an allosteric effector molecule. Alternatively, nicotine may induce hepatic CYP2B6 via enhance protein synthesis. Future experiments could be performed to determine whether nicotine acts to stabilizes and/or increase the translation of
hepatic CYP2B6. This can be assessed in vitro by pulse-chase experiments (Takahashi and Ono, 2003), where the rate of CYP2B6 protein synthesis and degradation can be tracked and compared in nicotine-treated and untreated cells.

In our study we did not observe a dose-dependent effect of ethanol on hepatic CYP2B6 protein levels. However, a previous study in rats, which tested a large range of ethanol doses (0.3-3.0 g/kg/day), demonstrated a dose-dependent effect of ethanol on hepatic CYP2B induction (Schoedel et al., 2001). In this same study, seven days of ethanol exposure (at a dose of 0.3 g/kg) was sufficient to induce CYP2B; however, it is not known how long protein levels stay elevated after ethanol treatment is stopped. Further studies are necessary to characterize the time course of hepatic CYP2B6 induction by ethanol.

1.2.2 Clinical consequence of elevated hepatic CYP2B6

Example 1 – Decreased therapeutic effect of efavirenz. Efavirenz is an anti-retroviral drug that is used as a therapy for HIV patients; it is inactivated mainly by CYP2B6 in the liver (Ward et al., 2003). Efavirenz has a relatively narrow therapeutic index, meaning small variation in efavirenz plasma levels can result in sub-therapeutic or toxic responses (Best et al., 2007). Efavirenz is taken orally and is subject to first pass metabolism, with a bioavailability of approximately 40-45% (Chiappetta et al., 2010). CYP2B6 genotype is a strong predictor of systemic exposure to efavirenz and therapeutic response (Frasco et al., 2012; Maimbo et al., 2012). Specifically, individuals classified as genetic CYP2B6 extensive metabolizers were shown to have lower efavirenz plasma levels and poorer therapeutic outcome compared to genetic CYP2B6 slow metabolizers (Frasco et al., 2012). Also, sub-therapeutic concentrations of efavirenz can result when it is co-administered with rifampicin, a known hepatic CYP2B6
inducer (López-Cortés et al., 2002; Yenny et al., 2011). Based on these findings, we hypothesize that the induction of hepatic CYP2B6 by chronic ethanol consumption would reduce systemic exposure to efavirenz and potentially lead to therapeutic failure. Alcohol use and abuse are prevalent in HIV patients and associated with poor response and more rapid progression to AIDS (Chander et al., 2006). To date, no studies have investigated the impact of chronic ethanol consumption on the pharmacokinetics of efavirenz, but they are certainly warranted. In addition to efavirenz, hepatic CYP2B6 is also involved in the metabolism of many clinical drugs (e.g., nevirapine, tamoxifen, bupropion, ketamine) (Table 5) and the induction of CYP2B6 by ethanol could potentially alter their therapeutic efficacy.

**Example 2 – Increased sensitivity to effects of cyclophosphamide**

Cyclophosphamide is chemotherapeutic pro-drug that is commonly used to treat a variety of cancers (de Jonge et al., 2005). To exert its cytotoxic effects, cyclophosphamide is metabolized mainly by hepatic CYP2B6 to 4-hydroxycyclophosphamide, which then undergoes further conversion to generate the active metabolites phosphoramide mustard and acrolein (de Jonge et al., 2005). Phosphoramide mustard mediates cyclophosphamide’s anti-cancer effects while acrolein can cause its toxic side effects (de Jonge et al., 2005). Since CYP2B6 is the rate-limiting enzyme for cyclophosphamide bioactivation (Roy et al., 1999), alterations in CYP2B6 activity may affect the therapeutic and toxic effects of cyclophosphamide.

Using a human primary hepatocyte-leukemia cell co-culture model, the selective induction of CYP2B6 in the hepatocytes via the activation of CAR resulted in enhanced anti-cancer effects of cyclophosphamide (Wang et al., 2013), suggesting that the induction of CYP2B6 by ethanol may increase the therapeutic potential of cyclophosphamide. Furthermore,
the CYP2B6*4 gene variant is associated with decreased cyclophosphamide metabolism in vitro (Ariyoshi et al., 2011) and patients homozygous for CYP2B6*4 have shorter progression-free survival when receiving cyclophosphamide treatment for various cancers (e.g. multiple myeloma, breast cancer) (de Jonge et al., 2005; Bray et al., 2010; Falk et al., 2012); this was attributed to reduced cyclophosphamide activation and thereby less effective treatment. Thus, reduced CYP2B6 activity can decrease cyclophosphamide activation and efficacy, suggesting that hepatic CYP2B6 induction by ethanol may increase the therapeutic effect of cyclophosphamide.

The use of cyclophosphamide is complicated by the occurrence of severe toxic side-effects, such as myelosuppression and hemorrhagic cystitis (Fraiser et al., 1991). The CYP2B6*6 gene variant is associated with a high rate of cyclophosphamide metabolism in vitro (Ariyoshi et al., 2011) and individuals who are homozygous for CYP2B6*6 have higher AUC ratios of 4-hydroxy-cyclophosphamide/cyclophosphamide and an increased risk for developing toxicity (Nakajima et al., 2007; Rocha et al., 2009). Therefore, although the induction of hepatic CYP2B6 may increase the anti-cancer effects of cyclophosphamide, ethanol-mediated hepatic CYP2B6 induction may also increased the risk for cyclophosphamide toxicity. In support of our hypotheses, the biotransformation of cyclophosphamide to 4-hydroxy-cyclophosphamide was more efficient in hepatocytes isolated from rats treated chronically with ethanol compared to hepatocytes from control rats (Neis et al., 1985). Hepatic CYP2B6 also plays an important role in the bioactivation of many other compounds (e.g. isofosfamide, aflatoxin B, NNK) (Table 5) and the induction of hepatic CYP2B6 by ethanol can potentially increase the therapeutic and/or toxic effects of these compounds as well.
1.3. Hepatic CYP2A6

1.3.1 Downregulation of hepatic CYP2A6 by nicotine

CYP2A6 is the principle hepatic enzyme responsible for nicotine inactivation to cotinine in humans (Nakajima et al., 1996a; Messina et al., 1997). Cigarette smoking can significantly decreased nicotine clearance in humans, which has been attributed to a decrease in hepatic CYP2A6 activity (Benowitz and Jacob, 2000). In our study, we demonstrated that chronic nicotine treatment can significantly decrease hepatic CYP2A6 protein levels and activity in AGM, leading to increased nicotine AUC and half-life (Chapter 2).

Nicotine treatment significantly reduced both hepatic CYP2A6 protein and mRNA in AGM, suggesting a decrease in transcription or enhancement of mRNA degradation (Chapter 2). Previous studies have also demonstrated nicotine-mediated downregulation of CYP2A6 protein and mRNA in AGM (Schoedel et al., 2003). Compared with our knowledge of hepatic CYP induction, the mechanisms involved in the suppression of hepatic CYPs are poorly understood. A potential mechanism for the downregulation of CYP2A6 by nicotine may involve dehydroepiandrosterone (DHEA), a steroid hormone secreted by the adrenal gland that is a precursor in the biosynthesis of other steroid hormones (androstenedione, androstenediol, testosterone, and estradiol) (Kroboth et al., 1999). DHEA treatment in rats is known to alter the expression of several hepatic CYPs, including the downregulation of CYP2C11 (Singleton et al., 1999; Ripp et al., 2003). The molecular events leading to the downregulation of CYP2C11 by DHEA are unclear. Nicotine exposure increases plasma DHEA levels in humans (Mendelson et al., 2007) and smokers have higher levels of DHEA compared to non-smokers (Field et al., 1994). It is possible that DHEA acts as negative regulator of hepatic CYP2A6 expression, and that nicotine causes a rise in DHEA plasma levels which leads to decreased hepatic CYP2A6 mRNA and protein levels. To further explore the impact of DHEA on CYP2A6, it would be
useful to examine the relationship between plasma DHEA and CYP2A6 activity in smokers and non-smokers. Also, studies assessing the effect of DHEA treatment on CYP2A levels, either in vitro or in animals models, would clarify whether DHEA can downregulate hepatic CYP2A6. Understanding the mechanism involved in CYP2A6 downregulation is important, as this pathway could potentially be targeted to develop novel drugs to aid with smoking cessation.

Inhibiting or reducing CYP2A6 activity is a potential strategy for smoking cessation (Sellers et al., 2003), based on the observations that individuals with reduced CYP2A6 activity smoke less cigarettes per day (Schoedel et al., 2004; Johnstone et al., 2006; Minematsu et al., 2006), smoke for shorter durations, (Schoedel et al., 2004) and have higher cessation success rates (Patterson et al., 2008; Ho et al., 2009).

It is unclear whether light smokers or non-daily smokers would have reduced hepatic CYP2A6 activity. A previous study in AGM demonstrated a 60% decrease in hepatic CYP2A6 using a 0.3 mg/kg bid nicotine dose for 18 days, which is similar to the 40% reduction in CYP2A6 protein observed in our study using a 0.5 mg/kg bid nicotine dose for 21 days. These results suggest that there is not a dose dependent effect of nicotine, at least within this dose range. Future studies are necessary to determine the dose and duration of nicotine exposure required to downregulate hepatic CYP2A6. Administration of a 21-mg transdermal nicotine patch twice daily for 10 days did not affect nicotine clearance in humans (Hukkanen et al., 2009). This is in contrast to our results and previous studies in monkeys and rodents showing that nicotine reduce hepatic CYP2A levels and/or activity (Stålhandske and Slanina, 1970; Schoedel et al., 2003). Similar to our discussion of the effect of transdermal nicotine patch on hepatic CYP2E1 (section 1.1.1 of discussion), it is possible that the peak plasma levels achieved from the nicotine patch may not be sufficient to stimulate the downregulation of CYP2A6.
Future studies should investigate the effect of nicotine route of administration on the downregulation of CYP2A6. It is also possible that 10 days of nicotine exposure is not sufficient to reduce hepatic CYP2A6 activity.

1.3.2 Clinical consequences of reduced hepatic CYP2A6

Example 1 – Decreased therapeutic response to tegafur. Tegafur is a chemotherapeutic pro-drug that is used for the treatment of hepatocellular cancer. It is bioactivated to 5-fluorouracil in the liver by CYP2A6 (Ikeda et al., 2000). 5-Fluorouracil plasma levels vary significantly among patients given a standard dose of tegafur (Walko and McLeod, 2008), and systemic exposure to 5-fluorouracil correlates with anti-tumour response (Milano et al., 1994). CYP2A6 slow metabolizers undergoing treatment with tegafur have decreased conversion rates of tegafur to 5-fluorouracil, decreased plasma 5-fluorouracil concentrations and poorer response rates (Park et al., 2011; Wang et al., 2011a). Given the positive association between CYP2A6 activity and tegafur efficacy (Park et al., 2011), the downregulation of hepatic CYP2A6 by nicotine exposure may decrease the therapeutic response to this drug. In our study, nicotine treatment was shown to reduce hepatic CYP2A6 in vitro activity by 55%, suggesting that the nicotine exposure could produce an effect similar to CYP2A6 slow metabolizers, who are hypothesized to have ≥ 50% reduction in CYP2A6 activity compared to wildtype (Malaiyandi et al., 2006; Benowitz et al., 2006b; Ho et al., 2009; Lerman et al., 2010). CYP2A6 also contributes to the metabolic inactivation of several other therapeutic drugs (e.g. letrazole, valproic acid) (Abu-Bakar et al., 2013). The downregulation of CYP2A6 by nicotine can potentially alter the therapeutic effect of these drugs.
Example 2 – Increased risk for coumarin hepatotoxicity. The downregulation of CYP2A6 by nicotine may also impact the toxic effect of certain compounds. For example, coumarin is an effective treatment for lymphedema, but is banned in some countries because of the incidence of idiosyncratic hepatotoxicity (Farinola and Piller, 2007). In humans, coumarin is mainly metabolized to 7-hydroxycoumarin by CYP2A6, but a small proportion of coumarin is metabolized by other CYPs to 3-hydroxycoumarin (Zhuo et al., 1999; Pelkonen et al., 2000). In contrast, rats metabolize coumarin predominantly to 3-hydroxycoumarin (Zhuo et al., 1999). The 3-hydroxylation of coumarin results in the formation of a 3,4-epoxide intermediate that is hepatotoxic. Rats are more susceptible to coumarin-induced hepatotoxicity compared to humans, most likely due to higher levels of 3,4-epoxide formation (Lake, 1999). Theoretically, in humans, inhibition of CYP2A6 could shift the metabolic balance towards 3,4-epoxide formation. It has been proposed that CYP2A6 poor metabolizers may be more susceptible to coumarin toxicity (Farinola and Piller, 2007). Similarly, smokers may also be at high risk for coumarin-induced toxicity, due to lower levels of hepatic CYP2A6 activity. We used coumarin as probe drug to assess in vitro hepatic CYP2A6 activity in our AGM study and found that nicotine significantly reduced coumarin metabolism to 7-hydroxycoumarin by 55%, providing evidence that nicotine can alter coumarin pharmacokinetics (Chapter 2).

2. The activity and regulation of CYPs in the brain

Total CYP levels in the brain are low compared to liver, therefore it is unlikely that brain CYPs substantially influence the overall pharmacokinetics of xenobiotics (Hedlund et al., 2001). However, the localization of brain CYPs to specific regions and cell types allows for a potentially considerable impact on metabolism in certain brain microenvironments and the brain.
as a whole (Miksys and Tyndale, 2012). There is growing interest in the function of CYPs in the brain, as their activity may be a crucial factor influencing the response to centrally-acting drugs and susceptibility to neurotoxins. The following section will discuss the activity and regulation of CYPs in the brain.

2.1 Activity of CYPs in the brain
There have been numerous in vitro studies demonstrating CYP-mediated metabolism of drugs, toxins and endogenous compounds in brain tissue (reviewed in Miksys and Tyndale, 2012). Previous studies in rats have demonstrated CYP2E1 activity (measured by chlorzoxazone 6-hydroxylation) and CYP2B activity (measured by 7-pentoxyresorufin dealkylation) in tissue from various brain regions (Dhawan et al., 1999; Wu et al., 2011). However, brain CYPs are more labile than hepatic CYPs and show reduced activity after freezer storage (Tyndale et al., 1999; Voirol et al., 2000), therefore in our study we focused on measuring protein levels of brain CYP2E1 and CYP2B6.

The demonstration of brain CYP function in vivo is challenging owing to the presence of substantial hepatic metabolism, which generates metabolites that can enter the brain from the periphery. However, our lab has recently developed techniques to detect and/or measure the enzymatic activity of brain CYPs in living animals (Miksys and Tyndale, 2009; Ferguson and Tyndale, 2011; Khokhar and Tyndale, 2011; Zhou et al., 2013). For example, brain CYP2B activity was assessed in rats by injecting a radiolabelled CYP2B mechanism based inhibitor ([3H]-8-methoxypsoralen) into the frontal cortex, where it was metabolized by CYP2B to a reactive metabolite that covalently bound to the active enzyme rendering it inactive and irreversibly radiolabelled. After sacrifice of the animals, radiolabelled CYP2B was retrieved.
from brain tissue indicating the presence of functional enzyme in the brain (Miksys and Tyndale, 2009). The selectivity of this method was demonstrated by (i) pretreatment with an injection of a non-radiolabelled CYP2B inhibitor, C8-xanthate, into one side of the brain, which significantly decreased the yield of [3H]-8-methoxypsoralen-radiolabelled CYP2B relative to the non-pretreated side and (ii) immunoprecipitation of the radiolabelled protein using an anti-CYP2B antibody. The enzymatic activity of brain CYP2E1 has not been assessed in vivo; however, future experiments can use radiolabelled CYP2E1-specific mechanism based inhibitors (e.g. diallyl sulphide or diethyldithiocarbamate) to labelled and measure functional brain CYP2E1 in situ (Brady et al., 1991b; Yamazaki et al., 1992).

Knowing that CYPs are active in the brain in vivo, research is now focused on demonstrating the functional role of brain CYPs. Several studies have shown that selective inhibition of CYPs in the brain can have a meaningful impact on the effects of centrally acting drugs and neurotoxins. For example, CYP2B was selectively inhibited in rat brain, but not liver, by administering a CYP2B mechanism-based inhibitor C8-xanthate via intracerebroventricular injection (Khokhar and Tyndale, 2011; 2012). Compared to untreated rats, the C8-xanthate-treated rats were more sensitive to the sedative effects of the anesthetic propofol (which is metabolically inactivated by CYP2B) and less susceptible to neurotoxicity from the pesticide chlorpyrifos (which is metabolically activated by CYP2B) (Khokhar and Tyndale, 2011; 2012). In a similar experiment CYP2D was selectively inhibited in rat brain by an intracerebroventricular injection of a CYP2D mechanism based inhibitor propranolol (Zhou et al., 2013). Compared to untreated rates, propranolol-treated rats exhibited decreased analgesia from codeine (which is metabolically activated to morphine by CYP2D) (Zhou et al., 2013).

These studies clearly demonstrate that CYPs have an important pharmacological and
toxicological role in the brain and alterations in brain CYP activity can have meaningful consequences for drug efficacy and neurotoxicity.

To better understand the functional consequences of CYP induction in the brain, animal models of brain specific CYP over-expression are needed. Chronic nicotine treatment can be used as a method to selectively induce CYP2B and CYP2D, in rat brain without affecting expression in the liver (Miksys et al., 2000; Yue et al., 2008). Although nicotine-treated rats can be a convenient and useful model for the selective overexpression of certain brain CYPs, it is important to remember that the magnitude and regional pattern of CYP induction by nicotine may differ from other brain CYP inducers such as ethanol. Another potential method for selectively over-expressing certain CYP isoforms in the brain could be via the virally delivery of transgenes. Recently this technique was used to overexpress alcohol dehydrogenase \textit{in vivo} in rat brain (Karahanian et al., 2011). An advantage of this method is that the CYP transgene could be delivered specifically to certain brain regions of interest (Karahanian et al., 2011).

\textbf{2.2 Regulation of CYPs in the brain}

The induction of brain CYPs is complex and can be organ-, region- and cell-specific. Currently, we have a very limited understanding of the the molecular mechanisms involved in the regulation of brain CYPs; however, speculation about potential mechanisms will be presented.

\textit{Organ-specific induction of brain CYPs}. Certain CYPs isoforms are regulated differently in the liver compared to the brain. For instance, CYP2D is essentially non-inducible in the liver, but brain CYP2D can be induced by compounds such as nicotine and the neuroleptic drug clozapine (Hedlund et al., 1996; Mann et al., 2008). In our study, we demonstrated the organ-specific
induction of CYP2B in the brain, but not the liver, by nicotine (Chapter 2 and 3). Previous studies in rats suggest that the induction of brain CYP2B by nicotine occurs via a transcriptional mechanism (Miksys et al., 2000). A potential mechanism for the selective induction of brain CYP2B by nicotine may involve the nuclear hormone receptor 4A (NR4A) subfamily. This subfamily of orphan receptors includes: neuron-derived clone 77 (Nur77; NR4A1; also known as NGFI-B), nuclear receptor related 1 (Nurr1; NR4A2) and neuron-derived orphan receptor 1 (NOR1; NR4A3) (Martínez-González and Badimon, 2005; Maxwell and Muscat, 2006), which are either exclusively or preferentially expressed in the brain (Watson and Milbrandt, 1990; Scearce et al., 1993; Mages et al., 1994; Maruyama et al., 1997). NR4A subfamily members can bind to a nuclear receptor-binding motif AGGTCA (Murphy, 1997) that is found within the 5’ region of the CYP2B6 gene in the PBREM (Honkakoski and Negishi, 1998). Chronic nicotine treatment upregulated Nur77 mRNA and protein in PC12 cells (Ichino et al., 2002), as well as Nur77 and Nurr1 mRNA in rat brain in vivo (Schochet et al., 2005; Saint-Preux et al., 2013). Therefore, members of the NR4A family may be involved in the brain specific induction of CYP2B6 by nicotine, due to their preferential expression in brain, their ability to bind to motifs within the CYP2B promoter region and their upregulation by nicotine treatment.

Region- and cell-specific induction of brain CYPs. In our study, CYP2E1 and CYP2B6 were both induced by ethanol and nicotine in a region- and cell-specific manner (Chapter 3). Given the large degree of cellular heterogeneity in the brain, it is not surprising that the effects ethanol and nicotine on CYP2E1 and CYP2B6 expression differ among regions and cell populations in the brain. The induction of CYPs in the brain can occur via transcriptional mechanisms (e.g. induction of brain CYP2B6 by nicotine) and post-transcription mechanisms (e.g. induction of
brain CYP2E1 by nicotine and brain CYP2B6 by ethanol) (Miksys et al., 2000) (Chapter 3). For CYPs that are induced via transcriptional activation, the region- and cell- specific induction may be due to variation in the expression of nuclear receptors, such as CAR, RXR, Nur77, Nurr1 and NOR1 among regions and/or cell types in the brain (Zetterström et al., 1996; Petersen et al., 2000; Lamba et al., 2004; Moreno et al., 2004). Brain CYPs could potentially be induced via protein stabilization and the region- and cell-specific pattern of induction may be due to the variation in proteasome activity and the expression of proteasome subunits/interacting proteins in different regions and cells in the brain (Zeng et al., 2005; Gorbea et al., 2006). If brain CYPs are induced via enhanced translational efficiency, the region- and cell-specific induction may be due to the differential expression of translation initiation factors and mRNA-binding protein among regions and cell types in the brain (Dagestad et al., 2006; Luchessi et al., 2008). Other mechanisms that may contribute to the regional and cell-specific induction of CYPs in the brain include differences in the post-translational modification of CYPs (e.g. phosphorylation or glycosylation state) as well as microRNA and epigenetic control of gene expression among different regions/cell types (Aguiar et al., 2005; Gomez and Ingelman-Sundberg, 2009; Klaassen et al., 2011; Rukov and Shomron, 2011).

3. Effects of ethanol and nicotine on brain CYPs

Interindividual variation in the activity of CYPs in the brain may contribute to observed difference in the sensitivity to centrally acting drugs and susceptibility to neurotoxicity. In our study we demonstrated the induction of CYP2E1 and CYP2B6 by ethanol and nicotine in the primate brain. In the following section, the region- and cell-specific regulation of CYP2E1 and CYP2B6 by ethanol and nicotine will be discussed. Also, some examples demonstrating the
potential consequences of elevated CYP2E1 and CYP2B6 in the brain will be presented.

3.1 Brain CYP2E1

3.1.1 Induction of brain CYP2E1 by ethanol and nicotine

Ethanol self-administration induced CYP2E1 in a region- and cell-specific manner in the primate brain (Chapter 3). As seen in Figure 34, the pattern of induced CYP2E1 in monkey brain resembles the pattern of elevated CYP2E1 that was observed in human alcoholics, suggesting that ethanol is responsible, at least in part, for the higher levels of CYP2E1 in the brains of human alcoholics.

Figure 34. Heat map showing the elevated levels of CYP2E1 protein in brain regions of human smokers, human alcoholics and monkeys exposed to ethanol and/or nicotine. For the human alcoholics the changes in CYP2E1 levels are relative to non-alcoholics; for the human smokers the changes in CYP2E1 levels are relative to non-smokers; for monkey exposed to ethanol and/or nicotine the changes in CYP2E1 levels are relative to control monkeys.
Chronic nicotine treatment induced monkey brain CYP2E1 in a region- and cell-specific manner in the primate brain. There is a substantial overlap between the brain regions where nicotine induced CYP2E1 in monkeys and regions where CYP2E1 was elevated in human smokers, suggesting that nicotine exposure contributes to the elevated levels of brain CYP2E1 protein in human smokers (Chapter 3) (Figure 34).

3.1.2 Clinical Significance of elevated CYP2E1 in the brain

**Example 1 – Increased risk for chronic solvent induced encephalopathy.** A number of organic solvents, such as n-hexane and trichloroethylene, are metabolized by CYP2E1 to reactive metabolites (Bolt et al., 2003). Long-term exposure to these solvents results in a disorder called chronic solvent-induced encephalopathy (CSE), which is characterized by permanent impairment to working memory, concentration and learning ability (Furu et al., 2012). Organic solvents are highly lipophilic and can readily distribute to the brain. Thus the bioactivation of solvents by CYP2E1 in the brain, at the target site for toxicity, is thought to be important for the development of CSE (Kezic et al., 2006). Individuals homozygous for the CYP2E1*5B high activity variant had a 6-fold increased risk for CSE compared to wild-type individuals (Kezic et al., 2006). Workers exposed to organic solvents who regularly consume alcohol (>14 drinks per week) were more likely to report CSE symptoms compared to solvent-exposed workers who did not consume alcohol (Cherry et al., 1992). Also, smoking was shown to modify risk for CSE via a gene-environment interaction (Ahmadi et al., 2002). Among smokers, individuals with a glutathione s-transferase M1 (GSTM1) null genotype are at increased risk for CSE, while in non-smokers, GSTM1 null subjects did not have elevated risk. GSTM1 is an enzyme involved in detoxification of reactive intermediates formed during cellular metabolism (Hayes and Pulford,
1995). Smokers with the *GSTM1* null phenotype may be more susceptible to CSE because they have reduced ability to detoxify the reactive metabolites produced from increased CYP2E1-mediated metabolism of organic solvents. Collectively, these findings suggest that alcohol consumption and/or smoking can increase risk for CSE, potentially via the induction of CYP2E1 in the liver and the brain. Further studies could be performed to determine the specific role of brain CYP2E1 in the pathology of CSE. This could be accomplished by selectively manipulating brain levels of CYP2E1 via pharmacological methods (i.e. administration of a CYP2E1 inhibitor specifically to the brain via a intracerebroventricular injection) or by a genetic/molecular techniques (brain-specific knockdown or overexpression of CYP2E1) and assessing the neurotoxic effect of solvents.

**Example 2 – Oxidative stress and damage in the brain.** The brain is particularly prone to damage from ROS because of its high oxygen demand, abundance of oxidizable polyunsaturated fatty acids and relatively limited anti-oxidant capacity compared to other tissues (Wang and Michaelis, 2010). The induction of CYP2E1 by ethanol in the brain has been associated with increased ROS and neurotoxicity (Zhong et al., 2012). In rats, chronic ethanol treatment can selectively caused neurotoxicity in the hippocampus, cerebellum and brainstem; CYP2E1 and ROS production were also selectively induced in these same brain regions and the severity of brain damage positively correlated with the level of CYP2E1 protein expression (Zhong et al., 2012). Furthermore, ethanol-induced neuroxicity in a human neuroblastoma cell line (IMR-32 cells) could be attenuated by pretreatment with a CYP2E1 inhibitor, suggesting that ethanol-mediated induction of CYP2E1 causes ROS production and neuronal damage (Zhong et al., 2012).
Alcoholism is associated with impairment of cognitive function, brain atrophy and neuronal losses (Meyerhoff et al., 2006). Two prominent morphological abnormalities observed in the brains of alcoholics are cerebellar atrophy and decreased Purkinje cell density, which manifest clinically as gait ataxia (Victor and Adams, 1959; Andersen, 2004). Even daily moderate ethanol consumption is also associated with decreased Purkinje cell density in the cerebellum (Karhunen et al., 1994). In our AGM study, ethanol selectively induced CYP2E1 protein in cerebellar Purkinje cells (Chapter 3), suggesting that the increased susceptibility of Purkinje cells to damage by ethanol may be a result of CYP2E1 induction. Smoking is also associated with atrophy in several brain regions including the frontal cortex and cerebellum, where nicotine induction of CYP2E1 occurs (Brody et al., 2004; Durazzo et al., 2012) (Chapter 3).

3.2 Brain CYP2B6

3.2.1 Induction of brain CYP2B6 by ethanol and nicotine

Chronic ethanol self-administration induced brain CYP2B6 in a region-and cell specific manner in primate brain. As seen in Figure 35, the pattern of ethanol-induced CYP2B6 in monkey brain resembles the pattern of elevated CYP2B6 that was observed in human alcoholics, suggesting that ethanol is responsible, at least in part, for higher levels of CYP2B6 in the brains of human alcoholics. In AGM, ethanol induced CYP2B6 in the frontal cortex, a region where CYP2B6 levels were not significantly elevated in human alcoholics. A possible explanation for this difference could be that the results from the AGM study, which was performed in a controlled setting, may simply be more robust than the results from the human association study which was conducted using a small number of human brain samples.
Figure 35. Heat map showing the elevated levels of CYP2B6 protein in brain regions of human smokers, human alcoholics and monkeys exposed to ethanol and/or nicotine. For the human alcoholics the changes in CYP2B6 levels are relative to non-alcoholics; for the human smokers the changes in CYP2B6 levels are relative to non-smokers; for monkey exposed to ethanol and/or nicotine the changes in CYP2B6 levels are relative to control monkeys.

Smoking is not associated with changes in hepatic CYP2B6 protein levels or activity (Hesse et al., 2004). Consistent with these results, nicotine did not affect hepatic CYP2B6 levels in monkeys (Chapter 2). Chronic nicotine treatment did induce monkey brain CYP2B6 in region- and cell-specific manner (Chapter 3). There is a substantial overlap between the brain regions where nicotine induced CYP2B6 in monkeys and regions where CYP2B6 was elevated in human smokers (Figure 35), suggesting that nicotine exposure contributes to the elevated levels of brain CYP2B6 protein in human smokers (Chapter 3). There are some apparent differences in the pattern of elevated CYP2B6 in the brains of nicotine-treated monkeys and human smokers. For example, human smokers have elevated CYP2B6 in the hippocampus and nicotine-treated
monkey do not. Also, in human smokers, several brain region show a significant increase in overall CYP2B6 protein content; however, in the nicotine-treated monkeys, the induction of CYP2B6 was observed in specific cell populations and did not significantly increased the overall levels of CYP2B6 in any of the regions assessed. These differences in expression of brain CYP2B6 between nicotine-treated monkeys and human smokers may indicate that there are differences between the effects of nicotine and cigarette smoke, and that additional compounds in cigarette smoke may regulate brain CYP2B6. It is also possible that brain CYP2B6 levels in smokers may be affected by other factors, such as alcohol use. In the human association study many of the smokers were also heavy drinkers, and the pattern of elevated CYP2B6 in smokers resembles the pattern of induced CYP2B6 in the monkeys exposed to both ethanol and nicotine (Figure 35). Alternatively, the dose and duration of nicotine administered to the monkeys (0.5 mg/kg bid for 21 days) may not have been sufficient to produce the elevation in brain CYP2B6 protein observed in human smokers who have been smoking heavily for years.

3.2.2 Clinical Significance

Example 1 – Decreased sensitivity to propofol. Propofol is a commonly used anesthetic that is inactivated to 4-hydroxypropofol by CYP2B6 (Court et al., 2001). As previously mentioned, alterations in the levels and activity of rat CYP2B in the brain can affect the local disposition and response to propofol (Khokhar and Tyndale, 2011). For example, rats were chronically treated with nicotine, which induces CYP2B in the rat brain but not the liver. Upon administration of the anesthetic propofol, nicotine-treated animals had reduced propofol-induced sleep time (Khokhar and Tyndale, 2011). Consistent with a role for brain CYP2B induction by nicotine in decreased propofol response, case reports reveal that smoking is associated with a higher
propofol dose requirement and reductions in post-operative nausea and vomiting (Chimbira and Sweeney, 2000). Likewise, alcoholics require a higher induction dose of propofol to induce anesthesia (Fassoulaki et al., 1993), possibly due ethanol-mediated induction of CYP2B6 and subsequent inactivation of propofol. Theses studies suggest that the induction of CYP2B6 by ethanol and/or nicotine in the brain can have a meaningful impact on the response to propofol and potentially other centrally acting drugs metabolized by CYP2B6 (e.g. bupropion, sertraline, methadone, nicotine).

**Example 2 – Increased susceptibility to chlorpyrifos neurotoxicity.** Chlorpyrifos is a widely used organophosphorus pesticide that produces neurotoxic effects via inhibition of acetylcholinesterase (Sultatos, 1994). To exert its toxic effect chlorpyrifos must be bioactivated by CYP2B6 to the potent cholinesterase inhibitor chlorpyrifos oxon (Tang et al., 2001).

Chlorpyrifos is mainly metabolized in liver; however, most chlorpyrifos oxon formed within the liver is rapidly detoxified before it can reach the systemic circulation (Sultatos et al., 1984). Therefore, extrahepatic sites of chlorpyrifos activation, such as the brain, are important in directly mediating the acute toxicity of chlorpyrifos (Sultatos et al., 1984). Manipulating the levels of rat CYP2B in the brain can significantly impact chlorpyrifos toxicity. For example, rats given intracerebroventricular injections of a CYP2B mechanism-based inhibitor that selectively inhibited CYP2B in the brain but not the liver had increased brain, but not plasma, chlorpyrifos levels, decreased brain chlorpyrifos-oxon levels, and attenuated neurotoxicity compared to controls (Khokhar and Tyndale, 2012). Thus, demonstrating a reduction in brain CYP2B6 can reduce the toxic effect of chlorpyrifos, supporting the contention that brain CYP2B6 induction by ethanol and/or nicotine exposure may potentiate toxic effect of chlorpyrifos. CYP2B6 also
metabolically activates many other environmental toxins such as the pesticide methyl parathion and the tobacco-specific procacinogen NNK, suggesting that ethanol and/or nicotine exposure may also increase the neurotoxic effects of these compounds as well (Code et al., 1997; Ellison et al., 2012).

4. Ethanol-mediated changes in nicotine pharmacokinetics

Chronic ethanol treatment significantly reduced the plasma nicotine $C_{\text{max}}$ (~70% decrease) and $\text{AUC}_{0-6\text{h}}$ (~50% decrease) achieved by AGM after a subcutaneous nicotine injection (Chapter 2). These ethanol-mediated changes in nicotine pharmacokinetics could not be explained by an increase in nicotine metabolism or the acute effects of ethanol (e.g., increased blood flow) (Chapter 2). We speculate that chronic ethanol exposure may affect the distribution kinetics of nicotine. Initial calculations suggest that monkeys exposed to ethanol had a noticeable, but nonsignificant, increase in the apparent $V_d$ of nicotine, whereas monkeys in the control and nicotine-only groups showed no change in nicotine $V_d$ over the course of the study. Our estimation of $V_d$ is based on the assumption of 100% nicotine bioavailability from a subcutaneous injection and pseudo-equilibrium conditions (Le Houezec et al., 1993); however, these assumptions may not be correct. To accurately assess ethanol-mediated changes in nicotine $V_d$, a study should be performed using intravenous nicotine administration, which eliminate variation in bioavailability, and also the kinetics of nicotine distribution from the central compartment (circulation) to the peripheral compartments (tissues) could be assessed.

In rats, chronic administration of ethanol increased the $V_d$ of procainamide, a drug that shares structural and chemical similarities with nicotine (Gole and Nagwekar, 1991), consistent with the possibility that ethanol can increase nicotine $V_d$. The potential effect of ethanol on
nicotine Vd may be explained by ethanol’s effect on cell membrane structure and permeability as well as epithelial barrier function (Nanji et al., 1994a; Bor et al., 1998; Carrasco et al., 2006; 2007). It is possible that chronic ethanol renders certain tissues more permeable to nicotine, thereby increasing nicotine Vd and decreasing nicotine levels in the plasma. Chronic ethanol treatment increased the partition coefficient (ratio drug concentration in tissue/drug concentration in blood) of procainamide in fat tissue, suggesting that ethanol can increase the permeability of procainamide, and possibly nicotine, into body fat (Gole and Nagwekar, 1991). Future studies could be performed to assess the effect of chronic ethanol treatment on the distribution of nicotine into various tissues. This can be achieved by comparing the steady-state tissue partition coefficient for nicotine in tissue from animals chronically treated with ethanol and control animals. Of particular interest is the effect of chronic ethanol exposure on the distribution of nicotine into the brain. An ethanol-mediated decrease in the distribution of nicotine to the brain may result in lower brain nicotine levels, increase the motivation to smoke, and contribute to the greater number of cigarettes/day smoked by individuals who regularly consume alcohol compared with those who do not (Witkiewitz et al., 2011). Dependent smokers who regularly consume alcohol may be adjusting their smoking behaviour (e.g. cigarettes/day) to maintain desired brain nicotine levels (Scherer, 1999).

5. Future directions
Throughout this chapter, ideas for future studies have been proposed. Summarized in this section are three main research themes for future research.
Theme 1 – Mechanistic studies to understand the molecular pathways involved in the regulation of hepatic and brain CYPs by ethanol and nicotine. Using cell-based assays or animal models, future studies can be performed to uncover the molecular mechanisms involved in the regulation of CYP2E1, CYP2B6 and CYP2A6 in the liver and/or brain. It is important to understand the molecular pathways that contribute to the induction or downregulation of CYPs because these pathways can be targeted pharmacologically, or by genetic techniques, to alter CYP expression for experimental or clinical purposes. Mechanistic studies investigating the regulation of CYPs can enhance our understanding of how organisms respond to drugs and toxins.

Theme 2 – Animal studies to determine the clinical impact of altered hepatic and brain CYPs. Some of the potential clinical consequences of altered CYP2E1, CYP2B6 and CYP2A6 activity in liver and/or brain have been presented in this chapter. Future studies could be performed in animals to demonstrate the impact of ethanol- and nicotine-mediated changes in CYP activity on the response to various xenobiotics. The animal models of selective brain CYP inhibition and induction that have been described (section 2.2 of discussion) can be used to explore the impact of altered brain CYPs expression on efficacy of therapeutic drugs, the reinforcing effect of drugs of abuse and susceptibility to toxicity.

For example, we have discussed the possibility that elevate levels of CYP2E1 in the brain may increased susceptibility to neurotoxins that are metabolically activated by CYP2E1, including certain organic solvents (section 3.1.2 of discussion). However, a causal relationship between elevated brain CYP2E1 levels and increased risk for neurotoxicity has not been demonstrated in vivo. Studying the impact of brain CYP2E1 induction in vivo is challenging,
since known inducers of brain CYP2E1 also increase hepatic CYP2E1 levels, and it is difficult to distinguish between the effects of brain vs. hepatic CYP2E1 induction. I propose the development of a rat model of brain-specific CYP2E1 overexpression to study the toxicological impact of elevated CYP2E1 levels in the brain. A lentiviral vector coding for rat CYP2E1 can be constructed and injected directly into rat brain to achieve a brain-specific increased in CYP2E1 levels. Mice that selectively overexpress CYP2E1 in the brain can then be exposed to neurotoxins that are bioactivated by CYP2E1 (e.g. trichloroethane, n-hexane) and behavioural screening tests can be used to assess the onset and severity of neurotoxicity in these animals compared to control rats (Euler et al., 2000).

Theme 3. Human association studies to establish a relationship between ethanol consumption and/or smoking and changes in the response to drugs or toxins. Ethanol and nicotine mediated alteration in the activity of CYP2E1, CYP2B6 and CYP2A6 levels in liver and/or brain can contribute the interindividual variability in drug response and susceptibility to toxicity. Epidemiological studies can be performed to investigate the relationship between alcohol use/smoking and the response to various xenobiotics that are metabolized by CYP2E1, CYP2B6 and CYP2E1. These types of studies will be facilitated by the increasing use of electronic medical records to manage patient information and medical history. Human association studies can also incorporate pharmacokinetic testing and brain imaging techniques to investigate the role of hepatic and/or brain CYP metabolism in mediating the relationship between alcohol use/smoking and response to xenobiotics.

For example, we have discussed the possibility that the induction of CYP2B6 by ethanol can affect the pharmacokinetics and efficacy of efavirenz (section 1.2.2) in discussion. I proposed a clinical study in HIV patients to examine the relationship between alcohol use and
efavirenz treatment outcomes. Patients undergoing efavirenz treatment can provide information about their frequency and quantity of alcohol consumption and these variables can be tested for associations with efavirenz treatment outcomes such as efavirenz plasma levels, CD4 cell count, and viral load. The influence of CYP2B6 genotype and drug compliance on efavirenz treatment outcomes should also be considered. The results from this study may help to improve the efficacy of efavirenz treatment by 1) establishing limits for alcohol use during efavirenz therapy and 2) suggesting dose adjustments or the use of alternative anti-viral drugs for chronic/dependent alcohol users.
Summary and conclusions

In conclusion, ethanol self-administration and nicotine treatment, alone and in combination, altered the levels of certain CYP2 family enzymes in AGM liver and/or brain, produced changes in CYP-mediated metabolism and affected drug disposition (summary in Table 18). The demonstration of these ethanol- and/or nicotine-mediated effects in non-human primates, strongly suggests that similar effects also occur in humans.

Table 18. Summary of results: Independent and combined effects of of ethanol and nicotine on CYP protein levels, *in vitro* activity and drug disposition

<table>
<thead>
<tr>
<th>Protein</th>
<th>In vitro activity in liver microsomes</th>
<th>In vivo drug disposition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Brain</td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>=</td>
<td>NA</td>
</tr>
<tr>
<td>Nicotine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>=</td>
<td>↑</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>↓</td>
<td>NA</td>
</tr>
<tr>
<td>Ethanol + Nicotine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>↓</td>
<td>NA</td>
</tr>
</tbody>
</table>

Key: ↑; increase, ↓; decrease, =; no change, NA; not assessed.

The induction of hepatic and brain CYPs by ethanol and nicotine may contribute to the interindvidual difference in drug response and susceptibility to toxicity. Exposure to ethanol and/or nicotine may prove to be an important predictor of response to certain xenobiotics. Now that we have established that ethanol and nicotine are inducers of specific CYP2 family isoforms
in primate liver and brain, future studies should focus on understanding the clinical impact of CYP induction or downregulation by ethanol and/or nicotine.
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