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Brain Drug-Metabolizing Cytochrome P450 Enzymes are Active *In Vivo*, Demonstrated by Mechanism-Based Enzyme Inhibition

Sharon Miksys*[1,2] and Rachel F Tyndale[1,2]

[1]Centre for Addiction and Mental Health, University of Toronto, Toronto, ON, Canada; [2]Department of Pharmacology, University of Toronto, Toronto, ON, Canada

Individuals vary in their response to centrally acting drugs, and this is not always predicted by drug plasma levels. Central metabolism by brain cytochromes P450 (CYPs) may contribute to interindividual variation in response to drugs. Brain CYPs have unique regional and cell-type expression and induction patterns, and they are regulated independently of their hepatic isoforms. *In vitro*, these enzymes can metabolize endogenous and xenobiotic substrates including centrally acting drugs, but there is no evidence to date of their *in vivo* function. This has been difficult to demonstrate in the presence of hepatically derived metabolites that may cross the blood–brain barrier. In addition, because of the membrane location of brain CYPs and the rate limiting effect of endogenous heme levels on the activity and appropriate membrane insertion of some induced CYPs, it has been unclear whether sufficient cofactors and coenzymes are present for constitutive and induced CYP forms to be enzymatically active. We have developed a method using a radiolabeled mechanism-based inhibitor of CYP2B1, ^{3}H-8-methoxypsoralen, to demonstrate for the first time that both the constitutive and induced forms of this enzyme are active *in situ* in the living rat brain. This methodology provides a novel approach to assess the function of enzymes in extrahepatic tissues, where expression levels are often low. Selective induction of metabolically active drug metabolizing enzymes in the brain may also provide ways to control prodrug activation in specific brain regions as a novel therapeutic avenue.
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INTRODUCTION

Many cytochrome P450 enzymes (CYPs) have tissue- and cell type-specific expressions and regulations, and the brain expresses its own unique complement of these enzymes (Miksys and Tyndale, 2002). These isozymes can metabolize a vast array of compounds including centrally acting drugs, neurotoxins, neurotransmitters, and neurosteroids (Ekins and Wrighton, 1999; Hiroi *et al*, 2001; Elbaz *et al*, 2004; Zanger *et al*, 2004; Seliskar and Rozman, 2007). Individuals respond differently to centrally acting therapeutic drugs, and their response is not always predicted by circulating drug levels in their plasma (Michels and Marzuk, 1993). Drugs that act on the central nervous system (CNS) may be metabolized *in situ* in the brain, and alterations in the degree of *in situ* metabolism may contribute to variation in an individual's response. The level of expression of brain CYPs is determined by an individual's genotype and also by exposure to environmental inducers and/or repressors.

Brain CYPs are more labile than their hepatic forms, and it has been difficult to demonstrate their enzymatic activities. Studies in artificial *in vitro* systems with added cofactors have shown that they have similar substrate specificity and *in vitro* kinetics to their hepatic forms (Forsyth and Chambers, 1989; Lin *et al*, 1992; Narimatsu *et al*, 1999; Tyndale *et al*, 1999; Coleman *et al*, 2000; Voirol *et al*, 2000). However, it has been difficult to demonstrate their function *in vivo* in the presence of extensive hepatic metabolism and the potential for metabolites to cross into the brain from the periphery. Brain CYPs are present in many different subcellular membrane compartments including plasma membrane, endoplasmic reticulum, Golgi, and mitochondria (Seliskar and Rozman, 2007). As such, it is not clear that there are sufficient necessary cofactors in close enough proximity for brain CYPs to be active *in vivo*. In the brain, endogenous heme levels have been shown to be rate limiting toward normal CYP function and appropriate membrane insertion, suggesting that basal and induced brain CYPs are not always functional (Meyer *et al*, 2002, 2005).

*Correspondence: Dr S Miksys, Department of Pharmacology, Rm 4334, University of Toronto, 1 King's College Circle, Toronto, ON, Canada M5S 1A8, Tel: +416 978 5155, Fax: +416 978 6395, E-mail: s.miksys@utoronto.ca
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Human CYP2B6 and the rat homolog CYP2B1 share 75% amino-acid identity and have overlapping substrate specificity, metabolizing a variety of substrates including drugs of abuse, neurotoxins, anticancer agents, anesthetics, and serotonin (Ekins and Wrighton 1999; Fradette *et al*, 2004). This enzyme is expressed variably among brain regions, and is inducible in the brain, but not in the liver, by nicotine, a constituent of tobacco smoke (Miksys *et al*, 2000). To demonstrate *in vivo* brain CYP2B1 activity, we took advantage of the enzyme's catalytic activity and used a radiolabeled mechanism-based inhibitor. These inhibitors are metabolized to reactive intermediates that bind covalently to the enzyme rendering it metabolically inactive. The furanocoumarin 8-methoxypsoralen (8-MOP) is a mechanism-based inhibitor of CYP2B1 with a K_I of 2.9 μM (Koenigs and Trager, 1998). Functional CYP2B1 metabolizes ^{3}H-8-MOP to a reactive ^{3}H-dihydro diol that covalently binds to the CYP2B1 apoprotein, inactivating the enzyme and irreversibly labeling it (Koenigs and Trager, 1998). The aims of this study were (1) to develop a method to demonstrate *in situ* metabolism by brain CYPs in a live animal by taking advantage of the enzyme's own catalytic ability and (2) to determine whether both the constitutive and induced forms of CYP2B1 are active *in vivo*.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250–300 g; Charles River, St Constant, QC, Canada) were housed in pairs with free access to food and water and maintained on a 12 h light–dark cycle. Nicotine-treated rats received 1 mg/kg nicotine base as nicotine bitartrate in saline, pH 7.4, subcutaneously once a day, for 7 days. This treatment paradigm is known to induce brain, but not hepatic, CYP2B1 (Miksys *et al*, 2000). Control animals received saline injections. All experimental procedures were carried out in accordance with the Canadian and NIH guidelines for the care and use of laboratory animals, and were approved by the University of Toronto's animal care committee.

In Vitro Incubations

Initial studies were carried out *in vitro* to assess feasibility, and how best to characterize the radiolabeled CYP2B1 protein. Incubation mixtures contained brain membranes, liver microsomes, or rat CYP2B1 expressed from cDNA in a baculovirus-insect cell system (Supersomes; BD Biosciences, Mississauga, ON, Canada). Rat liver microsomes and rat brain membranes were prepared as previously described (Miksys *et al*, 2000) from untreated animals, or from fresh tissue from saline- or nicotine-treated rats, and used immediately without freezing. Incubations were carried out for 20 min at 37°C with 50 mM Tris buffer (pH 7.4), 0 or 1 mM NADPH and 2.9 μM ^{3}H-8-MOP in a total volume of 125 μl.

Radiolabeled membrane-bound protein was retrieved by two methods. In the first method, reactions were terminated by the addition of 300 μl 20% trichloroacetic acid (TCA), incubated on ice for 30 min, and centrifuged for 1 min at 10 000*g*. The supernatant was discarded, and the pellet was washed with 500 μl ice-cold ethanol/ether (1:1), then centrifuged for 1 min at 10 000*g*. The supernatant was discarded, and the pellet was dried in the fume hood for 10 min then counted. In the second method, radiolabeled CYP2B1 membrane-bound protein was retrieved by immunoprecipitation. A 200 μl aliquot of monoclonal antibody in phosphate-buffered saline (pH 7.4, PBS) against rat CYP2B1/2 (Fitzgerald Industries, Concord, MA, USA) was added to the reaction mixture with 150 μg brain membranes, and incubated at 4°C overnight. The antibody–CYP2B1 protein complex was precipitated by incubating with gentle rocking at room temperature for 6 h with 500 μl of a 50% slurry of protein G immobilized on resin beads (Pierce, Rockford, IL, USA). The mixture was centrifuged for 1 min at 2500*g*, the supernatant was saved, the beads were washed twice with 500 μl PBS, centrifuged for 1 min at 2500*g*, and the wash supernatants were saved. The beads were reconstituted to a final volume of 1 ml with PBS, and a 100 μl aliquot was counted. Group means were compared by unpaired two-tailed Student's *t*-tests.

In Vivo Microinjection of Mechanism-Based Inhibitors

Animals were anesthetized with isoflurane, and placed in a stereotaxic frame. To assess the specificity of ^{3}H-8-MOP binding to CYP2B1, the left frontal cortex (Bregma coordinates dorsal–ventral −2.6, anterior–posterior +3.2, lateral +2.2 (Paxino and Watson, 1986)) was injected with 20 μg unlabeled C-8-xanthate in 0.5 μl sterile saline (over 1 min, and the Hamilton syringe removed after 3 min). C-8-xanthate is a highly selective mechanism-based inhibitor of CYP2B1 (Yanev *et al*, 2000). After 60 min, 10 μg ^{3}H-8-MOP in 0.5 μl sterile saline was injected (over 1 min, and the Hamilton syringe removed after 3 min) into the frontal cortex in both the left and right sides of the brain. After further 60 min the animal was killed, the frontal cortex was excised from around the injection sites (visualized by coinjection of Fast Green dye with C-8-xanthate), and a piece of occipital cortex was removed as a control.

Membranes were prepared, and an aliquot from each sample was counted. Radiolabeled CYP2B1 was retrieved from freshly prepared membranes by immunoprecipitation, as described above. For within-animal experiments, ^{3}H-8-MOP binding to CYP2B1 was compared between the side of the brain preinjected with C-8-xanthate and then ^{3}H-8-MOP, and the side of the brain injected with ^{3}H-8-MOP alone by paired two-tailed Student's *t*-tests. For between animal experiments, ^{3}H-MOP binding to CYP2B1 was compared between brains of nicotine and saline-treated rats by unpaired Student's *t*-tests.

The experimental time intervals were selected, in part, based on the diffusion rate of microinjected SCH 23 390 where the maximum diffusion distance over 120 min was 1.25 mm from the site of injection (Caine *et al*, 1995). To confirm that there was no contralateral diffusion of either ^{3}H-8-MOP or C-8-xanthate, we carried out two experiments. Firstly, we injected four rats with ^{3}H-8-MOP unilaterally, and killed the animals after 1 h. We prepared membranes from both contralateral and ipsilateral frontal cortex. The radioactivity detected in the contralateral frontal cortex was indistinguishable from baseline (55 ± 42 *vs* 39 d.p.m.), and was 0.5% of the radioactivity detected in membranes from

the ipsilateral frontal cortex (10 500 ± 900 d.p.m.). This indicates that there was no diffusion of ^{3}H-MOP to the contralateral frontal cortex after 1 h, the time frame used in these experiments. Secondly, we injected C-8-xanthate unilaterally, then after 1 h injected ^{3}H-8-MOP into the contralateral side only, and killed the animals after 1 h. There was no difference between the radioactivity retrieved from frontal cortex membranes from rats preinjected contralaterally previously with C-8-xanthate (10 300 ± 860 d.p.m.) and the radioactivity retrieved from frontal cortex membranes from animals injected unilaterally with ^{3}H-8-MOP but without preinjection with C-8-xanthate (10 700 ± 1200 d.p.m.). This indicates that there was no contralateral inhibition of ^{3}H-8-MOP binding, which infers that there was no contralateral diffusion of C-8-xanthate over 2 h. In addition, using increasing preinjection times of C-8-xanthate, we saw no reduction of the ^{3}H-8-MOP binding on the contralateral side. Together this strongly suggests that there was no significant contralateral diffusion of C-8-xanthate or ^{3}H-8-MOP.

Immunoblotting

Membrane proteins (50 μg) were separated by SDS–PAGE and immunoblotted as previously described (Miksys *et al*, 2000), except that polyclonal anti-CYP2B1/2 antibody (Fitzgerald Industries) was used for probing, and peroxidase-conjugated sheep anti-rabbit IgG (Millipore, Temecula, CA, USA) was used for detection. cDNA-expressed CYP2B1 (20 fmol; Supersomes, BD Biosciences) were loaded on each blot as a positive control. This antibody has been tested for cross-reactivity with several rat CYPs, 1A1/2, 2A1/2, 2C11, 2D1, 2E1, and 3A2. There was very weak cross-reactivity with CYP2C11, but this CYP had a higher mobility than CYP2B1/2, and was easily distinguishable on immunoblots. Samples were assayed four separate times, and films were analyzed by densitometry using MCID software (Interfocus Imaging Ltd, Linton, UK).

RESULTS

CYP2B1 Activity *In Vitro*

Increasing amounts of radiolabeled protein were retrieved by TCA precipitation from incubations with increasing amounts of liver microsomes, cDNA-expressed CYP2B1, and brain membranes. Negligible amounts of radiolabeled protein were detected in the absence of NADPH, indicating that enzymatic activation of the ^{3}H-8-MOP was necessary for protein labeling (Figure 1a). As proteins in addition to CYP2B1 may have been radiolabeled, CYP2B1 was immunoprecipitated from frontal cortex proteins with excess monoclonal anti-CYP2B1/2 (400 μl antibody/150 μg membrane protein; Figure 1b). In our previous studies we found that CYP2B2 mRNA and protein were almost undetectable in rat brain, and that there was no induction of CYP2B2 mRNA or protein by nicotine (Miksys *et al*, 2000). We are therefore confident that the radiolabeled protein immunoprecipitated by this antibody is primarily CYP2B1. Freshly prepared frontal cortex membranes were used, as we have previously found that brain CYP enzymatic activity is substantially reduced by freezing either tissue or

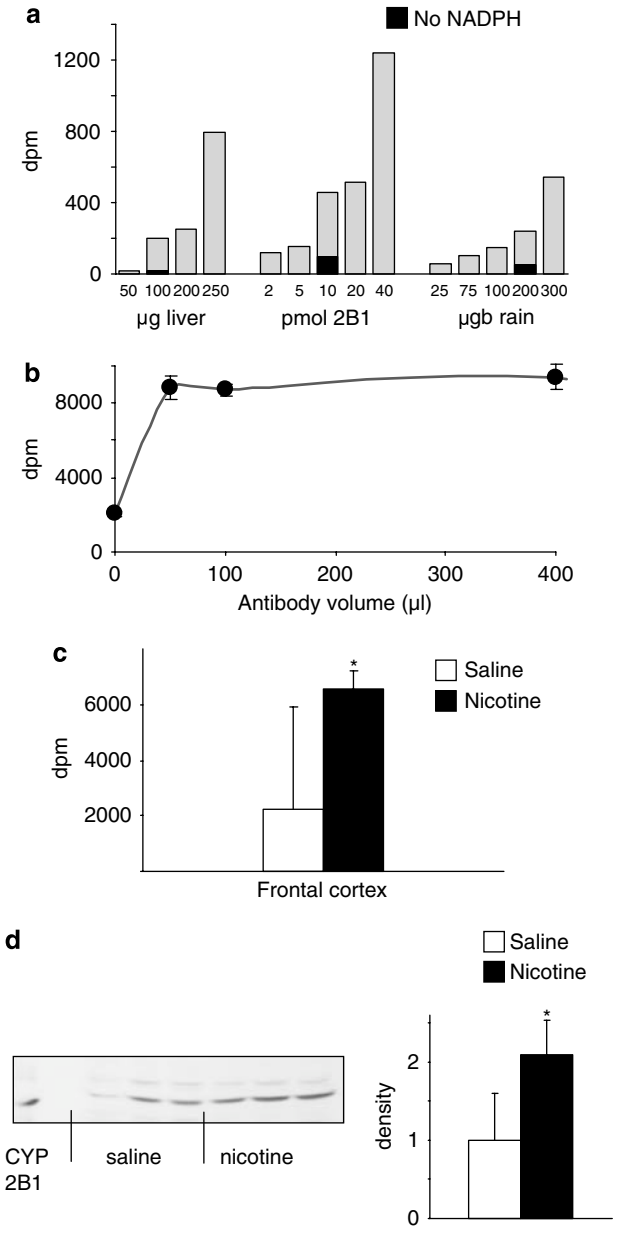

Figure 1 *In vitro* radiolabeling of membrane proteins. (a) TCA-precipitated membrane proteins from liver (left), baculovirus-insect cell CYP2B1 expression system (center), and brain (right) are radiolabeled only in the presence of NADPH. (b) Maximum immunoprecipitation of radiolabeled CYP2B1 occurs by 50 μl antibody and 37.5 μg frontal cortex membrane protein (mean ± SE of 2–3 experiments). (c) More radiolabeled CYP2B1 is immunoprecipitated from frontal cortex membranes from nicotine-treated than from saline-treated rats (mean + SD, 3 rats, $*p = 0.05$). (d) Immunoblotting shows more CYP2B1 in frontal cortex membranes of nicotine-treated rats (same animals illustrated in 1c, mean + SD, $*p = 0.03$).

membranes (Tyndale *et al*, 1999). There was three times more ($p = 0.05$) radiolabeled CYP2B1 in membranes from nicotine-treated rats than in membranes from saline-treated controls (Figure 1c), and immunoblotting of frontal cortex membranes from these animals detected twice as much CYP2B1 protein ($p = 0.03$) in nicotine-treated compared with saline-treated rats (Figure 1d). An additional, much weaker and higher mobility (approximately 70 kDa) band

was observed in all frontal cortex samples; this band was not affected by nicotine treatment. As all the known rat CYP2B proteins have molecular weights close to 50 kDa this band is unlikely to be a CYP2B protein, or a CYP (size range approximately 48–54 kDa) from another sub-family (Waxman and Walsh, 1982; Desrochers *et al*, 1996),

CYP2B1 Activity *In Vivo*

A substantial amount (32%) of the injected radiolabel was bound to frontal cortex membranes whereas no radioactivity was found in membranes from the occipital cortex (control tissue), confirming that the ^{3}H-8-MOP was confined to the vicinity of the injection site and not bound to CYP2B1 in nearby tissues. Pretreatment with the cold CYP2B1 inhibitor C-8-xanthate significantly reduced ^{3}H-8-MOP binding to brain membranes within each animal ($p < 0.001$, paired t-test, $n = 16$). Using immunoprecipitation with excess monoclonal antibody (400 µl/150 µg membrane), no radiolabeled CYP2B1 was detected in membranes from the side of the brain pretreated with C-8-xanthate (Figure 2a), suggesting complete inhibition of ^{3}H-8-MOP binding to CYP2B1. There was twice ($p = 0.004$, $n = 4$) as much radiolabeled CYP2B1 in frontal cortex of rats that had been treated for 7 days with nicotine compared with rats treated with saline (Figure 2b). Immunoblotting of these same tissues indicated a 1.5-fold increase ($p = 0.03$) in CYP2B1 protein levels in rats treated with nicotine compared to rats treated with saline (Figure 2c). Pretreatment with C-8-xanthate inhibited most of the CYP2B1-mediated metabolism of ^{3}H-8-MOP in both saline- (75%, $p = 0.004$, paired t-test) and nicotine- (90%, $p = 0.006$, paired t-test) treated rats (Figure 2b).

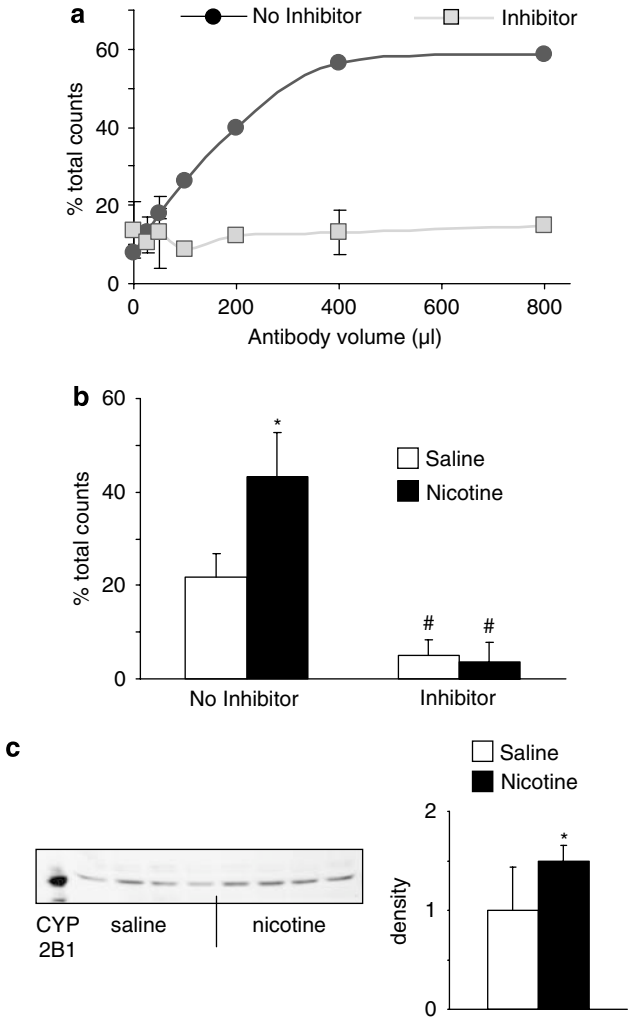

Figure 2 *In vivo* radiolabeling of brain CYP2B1. (a) Maximum immunoprecipitation of radiolabeled CYP2B1 occurs by 400 µl antibody and 150 µg frontal cortex membrane protein. Pretreatment with the inhibitor C-8-xanthate inhibits radiolabeling of immunoprecipitated CYP2B1 from frontal cortex membranes of rats treated with ^{3}H-8-MOP *in vivo* (mean ± SE, 3 experiments). (b) There is more radiolabeled CYP2B1 in frontal cortex of nicotine-treated rats compared to saline-treated rats in the absence of preinhibition (no inhibitor, $*p = 0.004$), and this difference is abolished by C-8-xanthate pretreatment (inhibitor) *in vivo*. There is significantly less radiolabeled CYP2B1 in the side of the brain pretreated with the inhibitor C-8-xanthate in both nicotine-treated and saline-treated rats (mean + SD, 4 rats/saline or nicotine group, $\#p < 0.005$). (c) Immunoblotting shows more CYP2B1 in frontal cortex membranes of nicotine-treated rats compared with saline-treated rats (same animals as illustrated in (b), mean + SD, $*p = 0.03$).

DISCUSSION

Brain CYPs are present at lower levels than their hepatic forms, but the brain is not a homogeneous organ, and in some regions and cells CYPs are expressed at levels as high as, or higher than, those in the liver (Miksys *et al*, 2000). These brain enzymes are unlikely to contribute to overall drug metabolism, however they may contribute to inter-individual variation in drug response through local *in situ* metabolism (Britto and Wedlund, 1992). Although brain CYPs have been shown to be enzymatically active *in vitro*, it is not clear that there are sufficient necessary cofactors and coenzymes in close enough proximity for them to be active *in vivo*, nor whether there is sufficient endogenous heme in the brain for induced brain CYPs to be correctly targeted and inserted into appropriate membranes, and to be functional (Meyer *et al*, 2002, 2005). Here we have shown for the first time that both constitutive and induced CYPs are enzymatically functional *in vivo*.

This technique takes advantage of the enzyme's catalytic ability. As we could not be sure that the mechanism-based inhibitor 8-MOP would remain selective for CYP2B1 at these subcellular concentrations, we increased the specificity of the assay at two levels. Firstly, we unilaterally injected a CYP2B1 inhibitor, C-8-xanthate (Yanev *et al*, 1999, 2000), in a within-animal design. Secondly, we assessed radiolabeled CYP2B1 by immunoprecipitation with a monoclonal antibody specific to CYP2B1. Together these two additional levels of selectivity increased our confidence that the assay detected enzymatically active CYP2B1.

These are the strongest supporting data reported yet for metabolism of centrally acting drugs within the live brain. Brain CYPs are highly inducible, and are often induced differently from their hepatic forms (Miksys and Tyndale 2004, 2006; Meyer *et al*, 2007). For example, nicotine induces rat CYP2B1 in the brain but not in the liver (Miksys *et al*, 2000), and ethanol induces rat CYP2B1 in the liver but not in the brain (Schoedel *et al*, 2001). There are several possible mechanisms of tissue-specific induction, including

tissue-specific expression of transcription factors, and/or tissue-specific expression of receptors that require activation for subsequent CYP induction. Alterations in brain CYP levels through genetic variation or through induction by commonly used drugs such as nicotine or ethanol would result in alterations in local CYP metabolism, which may contribute to the variation seen in efficacy, interactions, side effects, and toxicities of drugs that enter and act on the CNS (Gervasini *et al*, 2004). For example, brain CYP2B6 protein levels are higher in smokers compared to nonsmokers (Miksys *et al*, 2003), whereas hepatic levels are unchanged (Hesse *et al*, 2004), suggesting that smokers may have different therapeutic responses or side effects to CYP2B6 substrates. The anesthetic propofol is inactivated by CYP2B6, and there is evidence that smokers require a larger dose of propofol (Lysakowski *et al*, 2006) and report fewer postoperative side effects (Chimbira and Sweeney, 2000).

Alterations in brain CYPs that activate or inactivate neurotoxins may affect an individual's risk for neurotoxicity from a particular xenobiotic. In rats, phenobarbital treatment induces brain CYP2B1, and also potentiates the neurotoxic effects of the anticancer substrate 9-methoxy-*N*-(2)-methylellipticinium acetate (Upadhya *et al*, 2002); *in situ* neurotoxin activation may have implications in neurodegenerative diseases. CYP2D6 (McCann *et al*, 1997; Elbaz *et al*, 2004) has been implicated in Parkinson's disease; this enzyme may play a role in activating or inactivating putative toxins that are associated with Parkinson's disease (Suzuki *et al*, 1992; Gilham *et al*, 1997; Vaglini *et al*, 2004; Viaggi *et al*, 2007). Individuals with genetically deficient CYP2D6 are at an increased risk for Parkinson's disease, especially when exposed to environmental neurotoxins (Deng *et al*, 2004; Elbaz *et al*, 2004). Cigarette smoking reduces the risk for Parkinson's disease (Allam *et al*, 2004; Galanaud *et al*, 2005). CYP2D6 protein levels are higher in the brains of human smokers compared to nonsmokers (Miksys and Tyndale, 2004), suggesting that elevated brain CYP2D6 and consequent increased inactivation of neurotoxins may in part contribute to the neuroprotective effects of smoking against Parkinson's disease (Miksys and Tyndale, 2006).

There is growing interest in the role of CYPs in metabolism of endogenous neurochemicals, and a number of CYP-mediated pathways have been described. CYP2B6 biotransforms 5-hydroxytryptamine to hydroxylamine (Fradette *et al*, 2004), and metabolizes testosterone (Gervot *et al*, 1999). CYP2E1 contributes to the metabolism of arachidonic acid, fatty acids, and estrogenic metabolites (Lieber, 1999; Ohe *et al*, 2000). CYP2D6 metabolizes progesterone (Hiroi *et al*, 2001; Niwa *et al*, 2004) and biotransforms tyramine to dopamine (Hiroi *et al*, 1998; Niwa *et al*, 2004). CYP2D6 mediates several pathways in the cyclical metabolic interconvertions of endogenous indolethylamines, for example the regeneration of serotonin from 5-methoxytryptamine (Yu *et al*, 2003a, b). It is conceivable that alterations in levels of brain CYP enzymes and activity could cause subtle shifts in these endogenous neurochemical pathways, which could impact on neuropsychiatric conditions such as mood and overall mental health. Indeed, this may be a contributing factor to the clinical observations of personality differences between CYP2D6 extensive and poor metabolizers (Bertilsson *et al*, 1989; Llerena *et al*, 1993; Kirchheiner *et al*, 2006; Dorado *et al*, 2007), and between CYP2C19 extensive and poor metabolizers (Ishii *et al*, 2007; Yasui-Furukori *et al*, 2007).

This method of using a radiolabeled mechanism-based inhibitor to demonstrate enzyme activity *in vivo* is a novel and useful experimental tool for assessing enzyme function in all extrahepatic tissues where CYP expression levels may be low. It should prove particularly useful in investigating the effects of altered brain metabolism of drugs or endogenous neurochemicals on drug therapeutic efficacy, drug interactions, neurotoxicity, and behavior. Selective induction or inhibition of metabolically active drug-metabolizing enzymes in the brain may also provide ways to control prodrug activation in specific brain regions as a novel therapeutic avenue.

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DISCLOSURE/CONFLICTS OF INTEREST

Sharon Miksys has no financial interests to disclose. Rachel F Tyndale is a shareholder and chief scientific officer of Nicogen Inc., a company focused on the development of novel smoking cessation therapies; no funds were received from Nicogen for these studies, and the article was not reviewed by anyone associated with Nicogen before submission or revision.

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