Induction of the drug metabolizing enzyme CYP2D in monkey brain by chronic nicotine treatment

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Abstract

Cytochrome P450 (CYP) 2D6, an enzyme found in the liver and the brain, is involved in the metabolism of numerous centrally acting drugs (e.g. antidepressants, neuroleptics, opiates), endogenous neurochemicals (e.g. catecholamines) and in the inactivation of neurotoxins (e.g. pesticides, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)). Although CYP2D6 is essentially an uninducible enzyme in the liver, we show that smokers have higher CYP2D6 in the brain, especially in the basal ganglia. In order to determine whether nicotine, a component of cigarette smoke, could increase brain CYP2D, African Green monkeys were treated chronically with nicotine (0.05 mg/kg for 2 days, then 0.15 mg/kg for 2 days followed by 0.3 mg/kg for 18 days s.c., b.i.d.). Monkeys treated with nicotine showed significant induction of CYP2D in brain when compared to saline-treated animals as detected by western blotting and immunocytochemistry. No changes in liver CYP2D were observed in nicotine-treated monkeys. Induction was observed in various brain regions including those affected in Parkinson's disease (PD) such as substantia nigra (3-fold, $p = 0.01$), putamen (2.1-fold, $p = 0.001$) and brainstem (2.4-fold, $p = 0.001$), with the caudate nucleus approaching significance (1.6-fold, $p = 0.07$). Immunocytochemistry revealed that the expression of CYP2D in both saline- and nicotine-treated monkeys is cell-specific particularly in the cerebellum, frontal cortex and hippocampus. These results suggest that monkey brain expresses CYP2D, which is induced in specific cells and brain regions upon chronic nicotine treatment. Smokers, or those using nicotine treatment, may have higher levels of brain CYP2D6 that may result in altered localized CNS drug metabolism and inactivation of neurotoxins.

Keywords

CYP2D6; Nicotine; Smoking; Parkinson's disease; Brain drug metabolism; Non-human primate

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1. Introduction

Cytochrome P450 2D6 (CYP2D6) is an enzyme that metabolizes a range of endogenous (e.g. dopamine) and exogenous (e.g. parathion) substrates. It metabolizes approximately 25% of all clinically used drugs including analgesics (e.g. codeine), neuroleptics (e.g. clozapine), antidepressants (e.g. imipramine), and drugs of abuse (e.g. ecstasy) (Zanger et al., 2004). CYPs are expressed in a number of extrahepatic tissues including the brain. CYP2D has been identified in human (Miksys et al., 2002), dog (Tyndale et al., 1991) and rat (Miksys et al., 2000; Yue et al., 2008) brain tissue. Both, CYP2D mRNA and protein have been surveyed in the human brain (Miksys et al., 2002). Brain membranes from human (Voirol et al., 2000), dog (Tyndale et al., 1991) and rat (Tyndale et al., 1999), readily metabolize CYP2D6 probe drugs (e.g. dextromethorphan), indicating a functional enzyme. Furthermore, in rat brain both mRNA and protein correlate with activity (Miksys et al., 2000). A study demonstrating the initial analgesic effects of codeine due to its *in situ* metabolism to morphine is additional evidence of brain CYP2D function (Chen et al., 1990).

As CYP2D6 inactivates neurotoxins such as MPTP (Herraiz et al., 2006), tetrahydroisoquinoline (Suzuki et al., 1992), harmaline, harmine (Yu et al., 2003) and pesticides (Tang et al., 2002), individuals lacking a functional CYP2D6 may be more susceptible to neurotoxicity from these compounds. The expression and function of hepatic CYP2D6 is variable amongst individuals, because it is genetically highly polymorphic (<http://www.cypalleles.ki.se/cyp2d6.htm>). Functional CYP2D6 protein is absent in approximately 10% of Caucasians due to genetic polymorphisms. Population studies indicate that individuals with no functional alleles (i.e. *CYP2D6* poor metabolizers (PMs)) are at a higher risk for Parkinson's disease (PD) compared to those carrying one or two functional alleles (i.e. extensive metabolizers (EMs)) (McCann et al., 1997), and this risk increases with exposure to pesticides (Elbaz et al., 2004). Overall, these findings suggest that functional CYP2D6 has an important role in defense against endogenous and/or exogenous PD-causing neurotoxins.

Smokers are 50% less likely to develop PD (Alves et al., 2004). If CYP2D6 is higher in the brains of smokers it may contribute to the neuroprotection against PD observed in smokers. Therefore, we examined the levels of brain CYP2D6 in smokers and non-smokers. Nicotine, a component of cigarette smoke, has been shown in a Parkinsonian model to prevent the loss of dopaminergic markers and preserve neuronal plasticity (Quik et al., 2006a,b). It also reduces, and maintains the reduction in, the dyskinetic side effects of levodopa (Quik et al., 2007). Chronic nicotine treatment induces brain CYPs in rats (Howard et al., 2003; Yue et al., 2008). Therefore, we investigated if nicotine could induce CYP2D in a non-human primate brain and whether these changes were comparable to those seen in human smokers. Possible implications of induced brain CYP2D are that it may (a) provide neuroprotection from both endogenous and exogenous neurotoxins inactivated by CYP2D6, (b) alter localized CNS drug metabolism and drug response, and (c) influence mental health and behaviour through the altered metabolism of neurotransmitters and neurosteroids.

2. Materials and methods

2.1. Materials

Nicotine bitartrate was purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). All other chemical reagents were obtained from standard commercial sources. Protein assay dye reagent was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Pre-stained molecular weight protein markers were purchased from MBI Fermentas (Flamborough, ON, Canada). Nitrocellulose membrane was purchased from Pall Life Sciences (Pensacola, FL, USA). Human cDNA-expressed CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 were purchased from BD Gentest (BD Biosciences, Mississauga, Canada). Sheep polyclonal anti-human CYP2D6 antibody (PAb), against the C-terminal pentapeptide sequence of CYP2D6, and the synthetic immunogenic peptide were purchased from Biomol International (Plymouth Meeting, PA, USA). Rabbit polyclonal antiserum against amino acids 254–273 of CYP2D6 was generously provided by A. Cribb (PAb Cribb) and Merck & Co. (Whitehouse Station, NJ, USA) (Cribb et al. 1995). Horseradish peroxidase-conjugated anti-rabbit and anti-sheep secondary antibodies were purchased from Chemicon International, Inc. (Temecula, CA, USA). Biotinylated anti-mouse secondary antibody, avidinebiotin complex with peroxidase kit and 3,3'-diaminobenzidine (DAB) were purchased from Vector Laboratories (Burlington, ON, Canada). Chemiluminescent substrate was purchased from Pierce Chemical Company (Rockford, IL, USA). Autoradiographic film was purchased from Ultident Scientific (St. Laurent, PQ, Canada).

2.2. Human tissues

A subset of non-smoker and smoker individuals from our other study investigating human brain CYP2D6 (Miksys et al., 2002) was reassessed for CYP2D6 levels across several brain regions (Table 1). We have assayed additional samples (see Miksys et al., 2002 for methods) and reassessed the data subdivided by smoking status. Human brain tissues were obtained from the Brain Endowment Bank, University of Miami.

2.3. Animals

Young adult male African Green monkeys (*Chlorocebus aethiops*), were housed and treated at the Behavioural Sciences Foundation, Caribbean Primates Laboratory (St. Kitts). The saline-treated group ($n = 6$) received saline injections (s.c., b.i.d.) for 22 days. The nicotine-treated group ($n = 6$) received nicotine bitartrate injections (nicotine base in saline, pH 7.0, s.c., b.i.d) at 0.05 mg/kg for 2 days, then 0.15 mg/kg for 2 days followed by 0.3 mg/kg for 18 days. The final daily dose of nicotine administered to the monkeys (0.6 mg/kg, as 0.3 mg/kg b.i.d.) is similar to the average daily amount of nicotine (0.53 mg/kg) self-administered by a 70-kg smoker (Benowitz and Jacob, 1984). Peak plasma nicotine levels in human smokers range from 19–50 ng/ml (Schneider et al., 2001). In monkeys based on a 0.1 mg/kg pre-treatment nicotine challenge dose, 0.3 mg/kg twice daily dose was estimated to produce nicotine plasma levels higher than 20 ng/ml for approximately 19 h per day during nicotine treatment (Lee et al., 2006). Monkeys were given standard rations of Purina monkey chow, supplemented with fresh fruit and vegetables, and drinking water was available *ad libitum*. On day 22, 6 h after the morning drug injection, the animals were sacrificed under

ketamine anaesthesia. Organs were immediately dissected, one-half was flash frozen and stored at -80°C until further use, and the other half was fixed in 4% paraformaldehyde. The experimental protocol was reviewed and approved by the Institutional Review Board of the St. Kitts Behavioural Sciences Foundation and the University of Toronto Animal Care Committee. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

2.4. Immunocytochemistry

Immunocytochemical (ICC) CYP2D analysis was performed on frozen sections of fixed tissue as previously described (Lee et al., 2006). Frozen sections were blocked for 1 h in 1% w/v skimmed milk, 1% w/v BSA, 2% v/v normal horse serum (NHS) and 0.01% v/v Triton X-100 in PBS, and incubated for 48 h at 4°C in polyclonal anti-CYP2D6-peptide antiserum (PAb Cribb was diluted 1:500 in PBS with 0.1% BSA and 2% NHS). The antigen–antibody complex was visualized using biotinylated goat anti-rabbit immunoglobulin (diluted 1:500 in PBS) followed by the avidin–biotin complex technique and reaction with DAB and hydrogen peroxide. Negative control sections were incubated with pre-immune serum in place of antiserum. The levels of CYP2D were subjectively assigned by two independent individuals, blinded to treatment, as intense, strong, moderate, weak or absent.

2.5. Whole membrane preparation and protein assay

Human tissue membranes were isolated and prepared as previously described (Miksys et al., 2002). Monkey tissues from 12 brain regions were dissected by visual differentiation of the major brain regions based on brain atlases for the crab-eating macaque (*Macaca fascicularis*) (Martin and Bowden, 2000) and rhesus monkey (*Macaca mulatta*) (Snider and Lee, 1961) as previously described (Joshi and Tyndale, 2006; Lee et al., 2006). Briefly, brain sections were homogenized in 100 mM Tris, 0.1 mM EDTA, 0.1 mM DDT and 0.32 M sucrose (pH 7.4) and then centrifuged at $3000g$ for 10 min at 4°C . The supernatant was collected and the pellet resuspended and centrifuged again at $3000g$ for 10 min at 4°C . The combined supernatant was centrifuged at $110\,000g$ for 90 min at 4°C and the pellet resuspended in 100 mM Tris, 0.1 mM EDTA, 0.1 mM DDT, 1.15% w/v KCl and 20% v/v glycerol. The membranes were aliquoted and stored at -80°C . The protein content of brain membranes was assayed using the Bradford technique with a Bio-Rad Protein Assay kit.

2.6. Western blotting

Human brain membranes (50 μg) were separated on a 10% separating and 4.5% stacking SDS-PAGE. The proteins were transferred overnight onto nitrocellulose membranes and probed with polyclonal CYP2D6 antibody diluted 1:2000 with TBS-T followed by peroxidase-conjugated anti-rabbit secondary antibody diluted (1:3000). Monkey brain membranes were serially diluted to generate standard curves and to establish a linear detection range for the assay. Membrane proteins (10–60 μg) were separated by SDS-PAGE using a 4% stacking and 8% or 10% separating gel. To detect the CYP2D6-immunoreactive protein, described as monkey CYP2D, the membranes were incubated for 1 h in a blocking solution of 2% w/v skim milk powder or 2% gelatin, 0.1% BSA, 0.1% Triton X-100 and Tris buffer (TBS-T, 50 mM Tris, 150 mM NaCl, pH 7.4). The membranes were then incubated for 1 h at room temperature or overnight at 4°C with PAb diluted 1:1000 in 0.1% BSA in

TBS-T followed by three washes of 5 min each with TBS-T. Membranes were re-blocked with the same initial blocking solution for 45 min. The membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-sheep secondary antibody diluted 1:1500 in 0.1% BSA in TBS-T followed by three washes for 5 min each with TBS-T. Monkey CYP2D protein was then detected using chemiluminescence and exposure to autoradiographic film for 45 s–5 min. MCID Elite imaging software (Interfocus Imaging Ltd., Linton, UK) was used to analyze the films. To detect changes in brain CYP2D upon nicotine treatment, both saline- and nicotine-treated animals were run on the same western blot for each brain region. The saline distribution was determined by running all 12 brain regions on a single gel for each animal. Baseline corrections were made by subtracting the density of the film background from the band density.

For antibody purification with monkey liver, 80 µg (excess) of liver microsomes were separated in an 8% gel, transferred as described above, and membranes were incubated overnight with the PAb (1:1000) and washed 3 times for 5 min, as described above. The blots were then placed into elution buffer (200 mM glycine and 0.2% gelatin pH 2.8) for 10 min at 37 °C with agitation. Following elution the pH was adjusted to ~7 using 1 M Tris and the eluted PAb was used to probe membranes as described above. Peptide preabsorption was carried out by incubating the immunogenic peptide with PAb (1:1000) in TBS-T and 0.1% BSA at a 10:1 ratio (peptide:antibody) overnight at 4 °C prior to probing blots.

2.7. Statistical analysis

The comparison of brain CYP2D levels among brain regions of saline-treated monkeys was evaluated using one-way ANOVA followed by a Bonferroni's post-hoc test. The comparisons of brain region CYP2D protein levels between saline- and nicotine-treated monkeys were evaluated using a two-way ANOVA followed by testing each region between treatments using a least significant difference (LSD) post-hoc test. Results were expressed as mean and standard deviation (S.D.), which represent the average of six monkeys per group with the assay repeated 4–6 times. For substantia nigra tissue only four saline-treated animals, and for brainstem tissue only five saline- and five nicotine-treated animals, were available for assessment. Outliers were defined within a single western blot as $2 \times$ S.D. and removed from the analysis.

3. Results

3.1. Smokers have higher brain CYP2D6 than non-smokers

In humans, there were significantly higher levels of CYP2D6 in some brain regions of smokers compared to non-smokers (Fig. 1), such as the globus pallidus (7.9-fold), substantia nigra (3.5-fold) and the cerebellum (4.3-fold). This data should be interpreted cautiously due to the limited sample size. However, the observation of higher levels of CYP2D6 in specific brain regions of smokers provided the impetus for the subsequent studies using nicotine treatments.

3.2. CYP2D is expressed in the monkey brain

There was no cross-reactivity of PAb with cDNA-expressed CYP1A2, CYP2E1, CYP2B6, CYP3A4, CYP2C19 and CYP2A6, but there was minor cross-reactivity with CYP1A1 (Fig. 2A). CYP1A1 was detected with lower intensity despite the much higher (7 times) amount loaded compared to CYP2D6, and the migration of CYP1A1 was much faster than CYP2D6.

The PAb detected monkey CYP2D protein in both liver and brain. One CYP2D band was detected in the liver and two bands in monkey brain (Fig. 2B). Monkey liver purified PAb detected the same two bands (Fig. 2C right) as those detected by the unpurified antibody (Fig. 2C left) strongly suggesting that the CYP2D bands detected in brain are immunologically similar if not identical to monkey liver CYP2D. Preabsorption with the immunizing peptide completely abolished CYP2D immuno-detection in both the monkey liver and brain (Fig. 2C centre), indicating the specificity of the PAb for CYP2D6. The lower of the two bands co-migrated with monkey liver and cDNA-expressed CYP2D6 (Fig. 2B). Though some regions such as the CD had high expression of the top band, the overall expression was highly variable between saline-treated animals and was not significantly different among brain regions (ANOVA $p = 0.282$). Furthermore, the top band did not significantly differ between saline- and nicotine-treated groups for any brain region (2-way ANOVA $p = 0.521$, $Df = 1$, $F = 0.414$). For the purpose of this paper, only the bottom band is discussed as this band more closely resembles monkey liver CYP2D in terms of its migration and was affected by chronic nicotine treatment. Dilution curves were generated using brain regions from saline-treated animals including cerebellum, temporal cortex, thalamus, entorhinal cortex and occipital cortex. A representative dilution blot is illustrated in Fig. 2B and these dilution curves were used to determine the linear range of detection for the assay (Fig. 2D). A loading concentration of 25 μg was selected, so either a linear induction or reduction of brain CYP2D could be detected in the nicotine-treated group.

3.3. Expression patterns of brain CYP2D in saline-treated monkeys

The expression of CYP2D was measured by western blotting in the 12 brain regions examined from saline-treated monkeys (Fig. 3A,B). The CYP2D levels among different regions were highly variable (ANOVA, $p < 0.001$). There was a 10.8-fold difference between the highest and lowest CYP2D expressing regions, brainstem and entorhinal cortex, respectively. CYP2D in the brainstem was significantly higher than all regions ($p < 0.001$, Bonferroni post-hoc test).

ICC of brain sections showed cell-specific CYP2D expression in certain regions (Table 2 and Fig. 4). Specific cells in the hippocampus CA3 polymorphic layer lining the cerebral aqueduct, in the thalamus and in the pontine nucleus, showed moderate staining for CYP2D compared with the surrounding cells and tissue (Fig. 4). There was also some staining in Purkinje cells and in glial cells in the white matter of the cerebellum (Table 2 and Fig. 7D,F). Regions such as the hippocampus, which displayed very low levels of basal expression using western blotting (Fig. 3), showed moderate staining in some neurons using ICC (Fig. 4A), demonstrating the advantage of assessing the regional and cellular expression

of CYP2D in brain by both of these techniques. No staining was seen in sections of brain tissue when incubated with pre-immune serum as a control (Fig. 4E).

3.4. Induction of monkey brain CYP2D by chronic nicotine treatment

CYP2D levels were significantly different among brain regions in both the saline- and nicotine-treatment groups (2-way ANOVA; $p < 0.001$, Df = 11, $F = 48.00$) and between treatments ($p < 0.001$, Df = 1, $F = 26.84$). A significant interaction was also observed between treatment and brain region on CYP2D expression ($p < 0.001$, Df = 11, $F = 11.50$). CYP2D levels in the liver remained unchanged after chronic nicotine treatment (Fig. 5). Using the LSD post-hoc test we found that compared to saline-treated monkeys, CYP2D levels in nicotine-treated monkeys were significantly higher in the putamen (2.1-fold, $p = 0.001$), substantia nigra (3-fold, $p = 0.01$) and brainstem (2.4-fold, $p = 0.001$). The caudate nucleus also showed induction of CYP2D which approached significance (1.6-fold, $p = 0.07$). A significant decrease was observed in the parietal cortex (0.6-fold, $p = 0.01$) and thalamus (0.8-fold, $p = 0.02$). Other regions examined showed no significant differences in levels of CYP2D between saline- and nicotine-treated monkeys.

Induction of CYP2D by chronic nicotine in the substantia nigra was also observed using ICC where the neurons in the pars compacta and neuronal tracts in pars reticulata stained more intensely for CYP2D compared to saline (Table 2 and Fig. 6). Although the frontal cortex and cerebellum displayed no change in CYP2D levels with chronic nicotine treatment when assayed by western blotting (Fig. 5), the intensity of staining for CYP2D was higher in specific cells such as pyramidal neurons in layers III–V of the frontal cortex (Table 2 and Fig. 7B,C), and Purkinje cells and neurons in the deep cerebellar nuclei of the cerebellum (Table 2 and Fig. 7D–G) in nicotine-treated animals.

4. Discussion

This is the first study to show the induction of CYP2D in a non-human primate brain by a commonly used drug, nicotine. The induction of CYP2D by nicotine is brain region-specific and cell-specific (in select regions), with no change observed in liver. The induction within the brain is striking as CYP2D6 is considered uninducible in the liver by drugs and xenobiotics that readily induce other CYPs (e.g. phenobarbital) (Rae et al., 2001; Edwards et al., 2003).

Here we report that smokers have higher CYP2D6 in the brain, specifically in the basal ganglia, compared to non-smokers. We also show that nicotine, the most psychoactive agent in cigarette smoke, alone can induce CYP2D in primate brain. Of note, the induction is in similar regions to those where higher levels were observed in human smokers. This data strongly suggests that nicotine in cigarette smoke is responsible, at least in part, for the higher levels of brain CYP2D6 found in smokers. As induced CYP2D6 may be neuroprotective against PD, nicotine therapy may mimic the neuroprotective effects of smoking against PD.

We have described CYP2D6 expression and induction in a non-human primate brain. While rodents provide good models of some brain CYP expression and induction, they differ from

primates in many respects. Rats often have multiple forms of a CYP enzyme, such as CYP2D, and their brain is structurally different from that of primates. In our previous studies on brain expression and induction of other CYP enzymes, we have not always seen good concordance between rodents and primates, either monkeys or humans. It was therefore important to investigate CYP2D expression and induction by nicotine in a non-human primate, where we have control over drug, dosing and environmental influences, unlike in human postmortem brain studies. Nicotine metabolism is different between rats and primates. Monkeys metabolize nicotine in a similar manner to humans, via the CYP2A6-mediated pathway (Schoedel et al., 2003), while in rats nicotine is metabolized primarily by CYP2B1/2 (Nakayama et al., 1993). Furthermore, monkeys and humans respond similarly to neurotoxins (e.g. MPTP), and in PD models monkeys exhibit similar Parkinsonian symptoms to humans (Quik et al., 2006a,b, 2007).

Understanding the causes of altered levels of CYP2D in the brain and being able to mimic these alterations is important because this enzyme is involved in the inactivation of many neurotoxins and the metabolism of many CNS-acting drugs. Inhibition studies in liver microsomes indicate that CYP2D6 contributes up to 50% of MPTP inactivation (Coleman et al., 1996). In rat striatum, Vaglini et al. (2004) showed the localized inactivation of MPTP to PTP, a product generated largely by CYP2D. Neuroprotective effects against MPTP and MPP⁺ by CYP2D6 have been demonstrated in PC12 cells overexpressing this enzyme (Matoh et al., 2003). Data from female dark Agouti rats, which are deficient in CYP2D activity, provides evidence of increased susceptibility to the effects of neurotoxins such as MPTP. More pronounced effects of MPTP and longer reductions of motor activity were observed in dark Agouti rats compared to other strains (Jimenez-Jimenez et al., 1991). Human individuals genetically lacking a functional CYP2D6 are at increased risk for PD (McCann et al., 1997), perhaps due to the inability to inactivate PD-causing neurotoxins. Conversely, smokers have a reduced risk for developing PD, and nicotine, a component in smoke, is known to be neuroprotective in animal models of PD (Quik et al., 2006b).

CYP2D is expressed in the pigmented neurons of rat substantia nigra (Riedl et al., 1996, 1999), and is co-localized with tyrosine hydroxylase (Watts et al., 1998) and the dopamine transporter (Tyndale et al., 1991). The expression of CYP2D in the substantia nigra, in cells that degenerate in PD, indicates that the enzyme is ideally situated to inactivate PD-causing neurotoxins. Here, we show that smokers have higher CYP2D6 and that nicotine induces monkey brain CYP2D significantly in the regions affected in PD such as the substantia nigra. The increased levels of brain CYP2D6 in and around these specific brain regions may compete with neurotoxin activating enzymes (e.g. monoamine oxidase) and increase localized inactivation of neurotoxins, thereby protecting cells from damage by the reactive metabolite. Overall, this is important as it suggests that nicotine alone may be able to mimic the protection seen in smokers through this mechanism.

Epidemiological studies looking at CYP2D6 PM's risk for PD have not been stratified by smoking status (McCann et al., 1997). Since EM-smokers likely have higher levels of brain CYP2D6 compared to EM-non-smokers, there may be a substantial gene-environment risk interaction, such that PMs have increased relative risk compared to EM-non-smokers, who have higher risk than EM-smokers. Therefore, to obtain a better understanding of CYP2D6

influences in EMs, future association studies should attempt to stratify CYP2D6 EMs as smokers and non-smokers.

In addition to inactivating neurotoxins, CYP2D6 metabolizes a number of CNS-acting drugs and endogenous substrates. The region- and cell-specific localization of CYP2D in the brain may create metabolic microenvironments. Altered levels of CYP2D6 in these microenvironments may significantly influence localized drug metabolism and resulting drug response (Britto and Wedlund, 1992). There is evidence of very poor correlation between drug effect and plasma levels for neuroleptics and antidepressants (Michels and Marzuk, 1993). Localized CNS drug metabolism may contribute to this poor relationship, and this effect may be exacerbated in those exposed to cigarette smoke and/or on nicotine therapy, where they may experience altered therapeutic drug effect.

Accurately assessing brain CYP expression can be challenging knowing that (a) the brain is a very heterogeneous organ, (b) members of the CYP family have overlapping amino acid sequences and (c) polyclonal antibodies have multiplicity in epitope specificities towards their target (Ng et al., 2003). We developed a reliable assay which was both specific for detecting a CYP2D6-like protein in monkey brain, and was sensitive enough to detect low levels of this enzyme and changes following nicotine treatment. The detection of a single CYP2D band in monkey liver and multiple bands in the brain, by both unpurified and liver purified PAb, suggests possible expression of alternative forms of CYP2D in brain. These various forms may represent mRNA splice variants, post-translationally modified variants, or tissue-specific isoforms of CYP2D in the monkey brain. Functional brain-specific CYP2D iso-forms have previously been identified in rodent (e.g. CYP2D18) (Kawashima et al., 1996). Since there was no influence of nicotine treatment on the top band and no reduction in the top band when the bottom band was induced (e.g. caudate and putamen), this suggests a difference in regulation of the two CYP2D forms detected in monkey brain.

Studies across different species show that CYP2D is typically localized in specific brain regions and cells (e.g. pyramidal neurons in frontal cortex) (Norris et al., 1996; Miksys et al., 2000; Siegle et al., 2001). The brain region-, and cell-specific induction of CYPs by chronic nicotine treatment also occurs for monkey CYP2B6 (Lee et al., 2006) and CYP2E1 (Joshi and Tyndale, 2006) although the patterns of induction differ for each enzyme. Recently, our laboratory showed that chronic nicotine treatment significantly induced rat CYP2D protein in the striatum, cerebellum and hippocampus (Yue et al., 2008). The overall expression profile of human CYP2D6 (Miksys et al., 2002) is different from rodent brain (Yue et al., 2008), but to some extent resembles the patterns observed here in monkey brain. This further supports the use of a monkey model to study the expression/induction patterns of primate brain CYP2D6. No changes in mRNA levels were observed with nicotine treatment in rats, suggesting that the induction may be a result of post-transcriptional modification (Yue et al., 2008). Consistent with the lack of rat liver CYP2D induction by nicotine (Yue et al., 2008) and unchanged hepatic CYP2D6 in smokers (Funck-Brentano et al., 2005), no change in monkey liver CYP2D with nicotine treatment was observed. This suggests that CYP2D is differentially regulated in extrahepatic tissues, specifically in the brain. Some differences in CYP2D levels observed between human, monkey and rat brains may be due to species differences, difference in nicotine dosing regimens or routes of

administration (inhaling cigarette smoke vs. double/single bolus of nicotine s.c.) and/or the length of treatment (years vs. 22 days vs. 7 days).

In conclusion, brain CYP2D6 is higher in human smokers and nicotine induces brain CYP2D in monkey without altering hepatic CYP2D levels. The expression and induction of monkey brain CYP2D is region- and cell-specific, with the highest levels of induced CYP2D observed in the basal ganglia, including the substantia nigra. The localization of CYP2D in dopaminergic neuron rich regions, as well as in astrocytes makes this enzyme ideally situated to defend against neurotoxins and play a possible role in protecting against PD. In addition, the ability of nicotine to induce monkey brain CYP2D in a similar pattern to that observed in smokers may mean that nicotine can provide a healthier means of neuroprotection against PD than smoking. Altered levels of brain CYP2D may also significantly influence localized CNS drug metabolism and drug effect in a large portion of the population, which includes active and passive smokers, and those exposed to nicotine as a therapeutic.

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Fig. 1.

Human CYP2D6 levels are higher in some brain regions of smokers compared to non-smokers. The dotted line represents the relative level of brain CYP2D6 in non-smokers. There are significantly higher levels of CYP2D6 in smokers ($n = 10$) relative to non-smokers ($n = 3-16$) in the globus pallidus, substantia nigra and cerebellum (mean + SD; $*p = 0.04$, $**p = 0.02$, $***p = 0.008$ using Student's t -test). FC, frontal cortex; TC, temporal cortex; CG, cingulate gyrus; OC, occipital cortex; HC, hippocampus; EC, entorhinal cortex; CD, caudate nucleus; PT, putamen; NA, nucleus accumbens; GP, globus pallidus; SN, substantia nigra; Cv, cerebellar vermis; Ch, cerebellar hemisphere.

Fig. 2.

CYP2D6 is detectable and quantifiable in monkey brain. (A) A western blot testing the cross-reactivity of the PAb with cDNA-expressed CYPs. CYPs were loaded at 1 pmol, except CYP2D6 which was loaded at 0.15 pmol (7 times lower concentration than other CYPs); monkey liver was loaded at 5 μg. (B) A representative blot of increasing amount of monkey thalamus (TH) membrane used to generate a standard curve. This blot shows the comigration of the lower brain CYP2D6-immunoreactive protein (CYP2D) band with 5 μg monkey liver and 0.15 pmol of cDNA-expressed CYP2D6. (C) Three separate western blots (separated by the dotted lines) probed with: PAb in the absence of the immunogenic peptide (left), PAb in the presence of the immunogenic peptide (centre) and PAb purified using monkey liver (right) exposed for the same time. Monkey liver was loaded at 5 μg and caudate at 25 μg. In lane 3, 25 μg of caudate and 2.5 μg of liver were loaded together to

determine the comigration of brain and liver CYP2D as the amount of protein loaded or sample composition can alter migration of proteins in a high resolution gel. (D) A standard curve for the lower band of thalamus membrane, used to determine the linear range of detection and optimal loading amounts ($n = 3$ western blots).

Fig. 3.

Significant variation in expression of monkey brain CYP2D across 12 brain regions in saline-treated monkeys. (A) Brain CYP2D levels across various brain regions (mean – SD) from six saline-treated monkeys assessed by western blotting. BS had significantly higher levels of CYP2D than all other regions ($*p < 0.001$ Bonferroni post-hoc test). (B) A representative western blot of a saline-treated monkey (25 μ g of protein per region). FC, frontal cortex; TC, temporal cortex; PC, parietal cortex; OC, occipital cortex; HC hippocampus; EC, entorhinal cortex; CD, caudate; PT, putamen; TH, thalamus; SN, substantia nigra; CB, cerebellum; BS, brainstem.

Fig. 4.

Region- and cell-specific expression of CYP2D in saline-treated monkeys. (A) There was intense immunoreactivity seen in neurons (arrows) in the CA3 polymorphic layer in the hippocampus. (B) Cells lining the cerebral aqueduct (*) intensely stained for CYP2D. (C) Neurons in the thalamus (arrows) moderately immunostained for CYP2D. (D) Neurons and cell tracts in the pontine nucleus intensely immunostained for CYP2D. (E) No staining in absence of primary antibody. Bar: 100 μ m.

Fig. 5.

Induction of monkey brain CYP2D following chronic nicotine treatment. (A) A bar graph representing relative change in each region upon nicotine treatment. Mean levels – SD of CYP2D in nicotine-treated monkeys relative to the mean levels in saline-treated monkeys. The dotted line at 1 represents relative levels of brain CYP2D levels in saline-treated monkeys. There were significantly higher levels of CYP2D in nicotine-treated ($n = 4-6$) compared to saline-treated ($n = 5-6$) monkeys (LSD post-hoc test) in putamen (PT), substantia nigra (SN) and brainstem (BS). The caudate nucleus (CD) also showed induction of CYP2D that approached significance. There were significantly lower CYP2D levels in parietal cortex (PC) and thalamus (TH) in the nicotine-treated animals. There was no difference in monkey liver (LV) CYP2D between saline- and nicotine-treatments. Inset shows brain CYP2D levels (mean – SD) in saline- (white bars) and nicotine-treated (dark bars) monkeys. (B) Representative western blots from select brain regions. See Fig. 3 for brain region abbreviations; * $p = 0.07$, ** $p < 0.05$, *** $p = 0.001$.

Fig. 6. Higher CYP2D immunostaining in substantia nigra of nicotine-treated animals. Neurons in the substantia nigra pars compacta show less intense staining in saline-treated animals (A, arrow) compared to nicotine-treated animals (B, arrow). There was no staining observed in the absence of primary antibody (C). Cell tracts in the substantia nigra pars reticulata stained less intensely in saline-treated animals (D, arrow) compared to nicotine-treated animals (E, arrow). Bar: 100 μ m, for A–E.

Fig. 7.

Higher CYP2D immunostaining in frontal cortex and cerebellum of nicotine-treated animals. (A) No immunostaining of sections incubated without primary antibody. (B) There was no immunostaining of neurons in layers of frontal cortex, including pyramidal cells in layers III–V (inset), in saline-treated animals. (C) In nicotine-treated animals, neurons in layers II–VI of the frontal cortex showed strong CYP2D immunostaining, including pyramidal cells in layers III–V (inset). (D) There was weak immunostaining in granule cell (GCL), molecular (ML) and Purkinje cell (PC) layers but very little immunostaining in the white matter of cerebellum in saline-treated animals. (E) In nicotine-treated animals, there was more intense staining seen in Purkinje cells (arrow). There was slightly more immunostaining of small cells in the cerebellar white matter of nicotine-treated animals (G) compared to saline-treated animals (F). However in saline-treated animals there was very little staining of neurons in deep cerebellar nuclei (F inset), but strong staining of these cells in nicotine-treated animals (G inset). Bar: 50 μ m.

Table 1

Demographics, smoking status and genotype of tissue donors

Case	Age	PMI	Smoking	Genotype
C1	53	7.5	None	*1/*4
C3	46	13	None	*1/*4
C8	22	26	None	*1/*1
C9	35	18	None	*1/*4
C10	42	13	None	*1/*5
C11	39	24	None	*1/*4
C12	26	14	None	*1/*1
C13	17	15	None	*1/*5
C14	39	19	None	*1/*5
C15	25	13.5	None	*1/*1
C16	19	8.5	None	*1/*1
C17	37	14.5	None	*1/*4
C18	88	23.5	None	*1/*1
C19	65	18	None	*1/*1
C20	66	28	None	*1/*1
A1	58	10	None	*1/*1
C2	38	29.5	Yes ^a	*1/*4
C4	29	21	Yes ^a	*1/*1
C5	55	12	3–5 cig/d	*1/*1
A2	44	22	2 pk/d	*1/*4
A3	38	21	Yes ^a	*1/*5
A4	53	23	1 pk/d	*1/*1
A5	44	12	Yes ^a	*1/*4
A6	48	18	3 pk/d	*1/*5
A7	65	19	2 pk/d	*1/*4
A8	52	22	2–3 pk/d	*1/*1

^aUnknown number of cigarettes/day. Donor A6 was African American, donor A8 was a Caucasian female, all other donors were Caucasian males; 1 pk/d = 20 cigarettes (cig) smoked per day. PMI: postmortem interval (h); Pk/d: packs/day; Cig/d: cigarettes/day.

Table 2

Brain CYP2D protein immunocytochemical staining in saline- and chronic nicotine-treated monkeys

Brain region	Saline	Nicotine
Cortex		
Layer I	–	–
Layer II	–	++
Layers III–VI	–	+++
White matter	+ (Astrocytes)	+
Hippocampus		
Dentate gyrus	–	+
CA1-3		
Pyramidal cells	–	+
Polymorphic	++	++
Molecular	+	+
Subiculum II–IV	–	+
Entorhinal cortex II–IV	+	+
Caudate	++	+++
Putamen	++	+++
Nucleus accumbens	+	+
Globus pallidus		
Internal	++	++
External	++	++
Substantia nigra		
Pars compacta	++	++++
Pars reticulata	++	++++
Thalamus		
Ventral posterolateral		
Nucleus	++	++
Cerebellum		
Molecular layer	+	+
Purkinje cells	+	++++
Granule cell layer	+	+
White matter	–	–/+
Cerebellar nuclei neurons	+	+++
Brainstem		
Motor nuclei neurons	++	++
Aqueduct	+++	+++
Lateral geniculate nucleus	++	++

++++ Intense, +++strong, ++moderate, +weak, – no staining.