Brain CYP2D6 and its role in neuroprotection against Parkinson's disease

by

Amandeep Mann

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Abstract

The enzyme CYP2D6 can metabolize many centrally acting drugs and endogenous neural compounds (e.g. catecholamines); it can also inactivate neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1,2,3,4-tetrahydroisoquinoline (TIQ) and β-carbolines that have been associated with Parkinson's disease (PD). CYP2D6 is ideally situated in the brain to inactivate these neurotoxins. The *CYP2D6* gene is also highly polymorphic, which leads to large variation in substrate metabolism. Furthermore, *CYP2D6* genetically poor metabolizers are known to be at higher risk for developing PD, a risk that increases with exposure to pesticides. Conversely, smokers have a reduced risk for PD and smokers are suggested to have higher brain CYP2D6 levels. Our studies furthered the characterization and involvement of CYP2D6 in neuroprotection against PD. **METHODS:** We investigated the effects of CYP2D6 inhibition on MPP^+-induced cell death in SH-SHY5Y human neuroblastoma cells. We compared levels of brain CYP2D6, measured by western blotting, between human smokers and non-smokers, between African Green monkeys treated with saline or nicotine, and between PD cases and controls. In addition, we assessed changes in human brain CYP2D6 expression with age. **RESULTS:** Blocking CYP2D6 activity in SH-SY5Y cells with four diverse inhibitors significantly increased MPP^+-induced neurotoxicity. Smokers have higher brain CYP2D6 compared to non-smokers. In monkeys, basal expression of CYP2D6 varied across brain regions and was increased by chronic nicotine treatment in

select regions (notably the basal ganglia) and specific cell types. Expression of human brain CYP2D6 increased from fetal to 80 years of age in the frontal cortex; the influence of age on CYP2D6 expression was brain region specific. Compared to age-matched controls, PD cases had ~40% lower CYP2D6 levels in the frontal cortex, cerebellum and hippocampus consistent with lower CYP2D6 increasing the risk for PD. In the caudate and substantia nigra, CYP2D6 levels were similar between PD case and controls using Western blotting. This is likely due to the increase in CYP2D6-expressing astrocytes and much higher cellular CYP2D6 in PD affected areas as observed with immunocytochemical staining. **CONCLUSIONS:** Brain CYP2D6 can meaningfully inactivate neurotoxins, and it can be increased by nicotine in brain regions of interest to PD. These findings support the contention that higher brain CYP2D6 is protective and lower levels may contribute to increased risk for PD.

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Table of Contents

List of Table

List of figures

Summary of abbreviation

6-OHDA	6-hydroxydopamine
α-syn	α-synuclein
AGM	African Green monkey
AMHC	3-[2-(*N*,*N*-diethyl-*N*-methylammonium)ethyl]-7-hydroxy-4-methylcoumarin
AMMC	3-[2-(*N*,*N*-diethyl-*N*-methylammonium)-ethyl]-7-methoxy-4-methylcoumarin
AO	Aldehyde oxidase
BBB	Blood brain barrier
COUP-TFI	Chicken ovalbumin upstream promoter transcription factor I
CNS	Central nervous system
CYP	Cytochrome P450
CYP2D6	Cytochrome P450 2D6
DA	Dopamine
DAT	Dopamine transporter
EM	Extensive metabolizer
FMO	Flavin-containing monooxygenase
HNF-4α	Hepatocyte nuclear facto-4α
IM	Intermediate metabolizer
L-DOPA	*L*-3,4-dihydroxyphenylalanine
LB	Lewy body
LRRK	Leucine Rich Repeat Kinase
MAO	Monoamine oxidase
MBI	Mechanism based inhibitor
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nAChR	Nicotinic acetylcholine receptors
NO	Nitric oxide
Oct	Octamer transcription factor
PAb	Polyclonal antibody
PC12	Pheochromocytoma12
PD	Parkinson's disease
PINK	PTEN-induced kinase
PM	Poor metabolizer
ROS	Reactive oxygen species
SN	Substantia nigra
SNCA	α-synuclein gene
SNP	Single nucleotide polymorphism
Tg	Transgenic
UM	Ultra metabolizer

Section I: Introduction

Statement of Research Problem

Parkinson's disease (PD) is the second most common neurodegenerative disease in North America (Pahwa, 2003). It has a poorly characterized etiopathology; age, genetics, cigarette smoking, and exposure to environmental neurotoxins (e.g., pesticides) are all known to influence the risk of developing PD (Lang & Lozano, 1998; Grosset G. D., 2009). Recent research suggests interplay between genetic predisposition and exposure to environmental toxins influences the risk for PD (Vance *et al.*, 2010). In light of this research, enzymes are good candidates for study of the etiopathology of PD, as enzymes can metabolize environmental neurotoxins and alter their effects in the brain. Of particular interest to us is the xenobiotic metabolizing enzyme Cytochrome P450 2D6 (CYP2D6) that has been implicated in the etiopathology of PD (McCann *et al.*, 1997; Elbaz *et al.*, 2004).

CYP2D6 can inactivate many of the neurotoxins that have been associated with PD or PD-like pathology, including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1,2,3,4-tertrahydroisoquinoline (TIQ), and β-carbolines (Suzuki *et al.*, 1992; Modi *et al.*, 1997; Yu *et al.*, 2003d). CYP2D6 is expressed with relatively high levels in the basal ganglia, a brain structure that is predominantly affected in PD, and is, therefore, ideally situated to locally inactivate neurotoxins in the brain (Tyndale *et al.*, 1999; Miksys *et al.*, 2002; Bromek *et al.* 2010). These findings led up to investigate whether CYP2D6 is involved in neuroprotection in PD.

In contrast to environmental factors that increase the risk for PD (e.g., toxins, pesticides), cigarette smoking protects against developing PD (Alves *et al.*, 2004). Nicotine, a

component of cigarette smoke, has been shown to be neuroprotective in models of toxin-induced PD (Quik & Kulak, 2002). Over-expression of CYP2D6 in PC12 cells protects against cytotoxicity induced by 1-methyl-4-phenylpyridinium (MPP^+), the neurotoxic metabolite of MPTP (Matoh *et al.*, 2003). Notably, nicotine has been shown to induce brain CYP2D in rats and mice (Miksys & Tyndale, 2006; Yue *et al.*, 2008; Singh *et al.*, 2009). Currently, there are no studies that have shown whether cigarette smoking and nicotine can alter brain CYP2D expression in primates. However, if it can be determined that brain CYP2D6 expression is induced by nicotine, and is higher in cigarette smokers, the results would support the theory that elevated levels of brain CYP2D6 may be neuroprotective against PD.

Certain polymorphisms in *CYP2D6* that result in non-functional or reduced enzymatic activity have been associated with an increased risk for PD (McCann *et al.*, 1997; Singh *et al.*, 2010). Individuals who are exposed to certain neurotoxins (e.g., pesticides), and who are *CYP2D6* poor metabolizers, have further increased risk of developing PD (Deng *et al.*, 2004; Elbaz *et al.*, 2004). To date, only the *CYP2D6* genetic predisposition to PD has been studied. Comparisons between CYP2D6 protein levels in PD and control brain tissue samples remain to be done. This study will ascertain whether low brain CYP2D6 protein expression is associated with higher susceptibility to PD-causing neurotoxins and increased risk for PD.

Review of the literature

1. Cytochromes P450 2D6 (CYP2D6)

1.1 Cytochromes P450

Cytochromes P450 (CYPs) are a family of heme-containing metalloenzymes actively involved in the oxidation of a diverse array of substrates (Estabrook, 1999; Coon, 2005). CYP substrates range from endogenous compounds, such as steroids and neurotransmitters, to exogenous chemicals, such as clinical drugs and environmental toxins (Rendic & Di Carlo, 1997). The broad range of substrate specificity is largely due to the diversity of CYPs. In humans, CYPs are divided into 18 families that share ~40% amino acid homology and 57 subfamilies that share ~55% amino acid homology (Nelson, 2006). Among the 18 human CYP families, CYP1, CYP2, and CYP3 are responsible for the biotransformation of many clinical drugs and other xenobiotics in humans (Emoto *et al.*, 2006; Zanger *et al.*, 2008). The CYP2 family is the largest of the three, incorporating 16 full-length gene members (Lewis, 1998; Hoffman *et al.*, 2001).

In the liver, the overall level of expression of CYPs varies across different family members (**Figure 1A**). For example, CYP3A4 is the most abundantly expressed CYP, accounting for ~30% of the total CYP content. In contrast, CYP2D6 accounts for only ~5% of the total CYP content (Guengerich, 2003; Emoto *et al.*, 2006). The expression level of a CYP and its contribution to substrate metabolism may differ because of genetic variation and/or exposure to environmental factors, such as inducers (e.g., phenobarbital for CYP2B6) or inhibitors (e.g., quinidine for CYP2D6) (Lee *et al.*, 2006b; Ai *et al.*, 2009). Five members of the CYP superfamily are most actively involved in the metabolism of clinical drugs. These five CYPs include, in order from the greatest contribution to the least contribution to the biotransformation of most common clinical drugs, CYP3A4, CYP2D6, CYP2C9, CYP2C19,

and CYP1A2 (**Figure 1B**) (Guengerich, 2003; Emoto *et al.*, 2006). CYP2D6, though expressed at much lower levels than other CYPs (e.g., CYP2C9), is the second most important drug metabolizing enzyme and contributes to the metabolism of ~30% of clinically prescribed drugs (Zanger *et al.*, 2004).

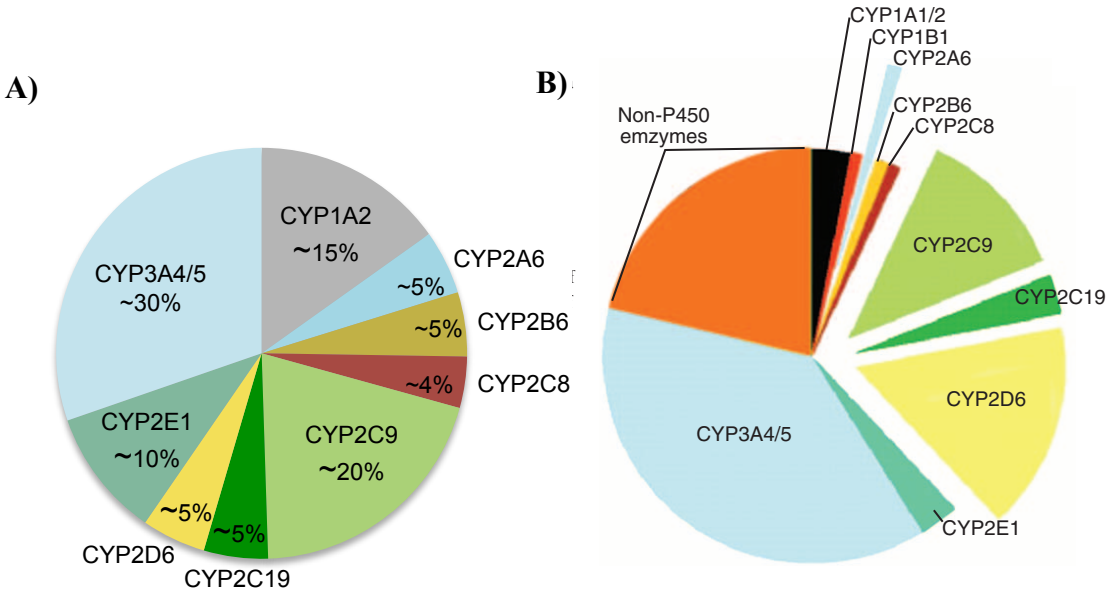


Figure 1: CYP hepatic expression and contribution to drug metabolism
A) Hepatic CYP expression as a percentage of total CYP content (Rendic & Di Carlo, 1997; Emoto *et al.*, 2006). **B)** Contribution of CYPs to metabolism of clinical drugs, adapted from Guengerich (2003) by permission from the copyright holder.

1.2 CYP2D6 substrates

CYP2D6 substrates tend to be lipophilic bases with a planar aromatic ring and a nitrogen atom that can be protonated at physiological pH (de Groot *et al.*, 2009; Wang *et al.*, 2009). CYP2D6 substrates include a variety of both exogenous (**Table 1**) and endogenous compounds (discussed in **Section 2.2.B**) that can either be metabolically activated (e.g., codeine) or inactivated (e.g., desipramine) by CYP2D6 (Zanger *et al.*, 2004). The promiscuity of CYP2D6 is exemplified by its ability to metabolize neurosteroids, biogenic amines,

Table 1: Examples of exogenous CYP2D6 substrates

Clinical drugs		Recreational drugs	Toxins
Analgesics/antitussives	**Antidepressants**	Paramethoxyamphetamine	MPTP
Codeine	Desipramine	Amphetamine	TIQ
Dextromethorphan	Imipramine	MDMA	β-carbolines
Hydrocodone	Nortriptyline	MDME	Diuron
Norcodeine	Fluoxetine	MDEA	Antifouling biocide
Oxycodone	Paroxetine	MDA	Parathion
Antipsychotics	Citalopram	MPPP	Chlorpyrifos
Haloperidol	Mirtazapine	MPBP	Diazion
Risperidone	**β-adrenergic blocking agent**		Carbamate
Perphenazine	Bufuralol		Atrazine
Thioridazine	Metoprolol		Imidaclopride
MAO-inhibitor	Propranolol		NNK
Amiflamine	Timolol		Ortho-phenylphenol
Brofaromine	**Antiarrhythmics**		
Antihypertensive	Propafenone		
Debrisoquine	Sparteine		
	Calcium antagonist		
	Perhexiline		

MDMA, 3,4-methylenedioxymethamphetamin; MDME, 4-methylenedioxyamphet- amine; MDEA 3,4-methylenedioxy-N-ethylamphetamine; MDA, methylenedioxyamphetamine; MPPP, 1-methyl-4-phenyl-4-propionoxypiperidine; MPBP, 4'-methyl-alpha-pyrrolidinobutyrophenone; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Rendic & Di Carlo, 1997; Pritzker *et al.*, 2002; Zanger *et al.*, 2004; Peters *et al.*, 2008; Sauer *et al.*, 2009; Wang *et al.*, 2009).

pesticides, neurotoxins, and a range of clinical drug classes (**Table 1)**. Other therapeutic drug classes which are CYP2D6 substrates, but not listed in **Table 1**, include appetite suppressants, calcium antagonists, vasodilators, anti-diabetic drugs, anti-dementia drugs, antiestrogens, antiemetics, anti-attention deficit/hyperactivity disorder drugs, and antihistamines (Rendic, 2002; Zanger *et al.*, 2004).

The majority of the aforementioned substrates are also metabolized by other CYPs to some degree. Select substrates that primarily (≥80%) undergo a specific metabolic reaction catalyzed by a single CYP are called probe drugs (Frank *et al.*, 2007). The resulting parent and metabolite ratio from these reactions serves as good phenotypic markers to assess levels of CYP-specific activity. Commonly used CYP2D6 probe drugs include debrisoquine, sparteine, dextromethorphan, bufuralol, and, to a lesser extent, tramadol and metoprolol (Komura & Iwaki, 2005b; Zhou, 2009).

The β-adrenergic receptor blocking agents, metoprolol, timolol, and propranolol (all used in **Chapter 1**), are selective substrates with a high affinity for CYP2D6 (Yamamoto *et al.*, 2003). Metoprolol, a β_1-adernergic receptor blocker, is used as a treatment for hypertension, angina pectoris, cardiac arrhythmias, and chronic heart failure (Prakash & Markham, 2000; Komura & Iwaki, 2005a). Metoprolol is α-hydroxylated almost entirely by CYP2D6 (K_m=21 μM), but can also undergo *O*-demethylation by CYP2D6 (Madani *et al.*, 1999; Komura & Iwaki, 2005a). Propranolol, a non-selective β-adrenergic receptor blocker, is used to treat hypertension, angina pectoris, and cardiac arrhythmias (Komura & Iwaki, 2005a). Propranolol is hydroxylated largely (>60%) by CYP2D6 (K_m=3.9 μM) to 4-hydroxypropranolol (Ward *et al.*, 1989; Komura & Iwaki, 2005b). Although, CYP1A2 (K_m=21.2 μM) can also hydroxylate propranolol, CYP2D6 has a lower affinity and a higher substrate turnover (V_{max}) for propranolol than does CYP1A2. As a result, CYP2D6 contributes to a greater degree of 4-

hydroxypropranolol formations (Johnson *et al.*, 2000). Timolol, another non-selective β-blocker, is used mainly as a topical treatment for glaucoma and ocular hypertension (Wood *et al.*, 2003). Timolol is primarily (>90%) hydroxylated by CYP2D6 (K_m= 7 μM), but CYP2C19 also plays a minor role (<10%) in its hydroxylation (Volotinen *et al.*, 2007).

3-[2-(*N*,*N*-Diethyl-*N*-methylammonium)-ethyl]-7-methoxy-4-methylcoumarin (AMMC) is a newly designed probe substrate of CYP2D6 that is primarily used for *in vitro* high-throughput screening. This non-fluorescent compound is primarily *O*-demethylated by CYP2D6 (K_m=3 μM) to the fluorescent metabolite 3-[2-(*N*, *N*-diethyl-*N*-methylammonium) ethyl]-7-hydroxy-4-methylcoumarin (AMHC) (Chauret *et al.*, 2001; Ghosal *et al.*, 2003). The CYP2D6 mediated *O*-demethylation of AMMC correlates strongly (r^2=0.95) with the 1'-hydroxylation of bufuralol, a selective CYP2D6 probe drug (Chauret *et al.*, 2001). These results indicate that, similar to the previously used CYP2D6 probe substrate bufuralol, AMMC is also a reliable probe substrate for CYP2D6 activity *in vitro*.

1.3 CYP2D6 inhibitors

There are different forms of CYP inhibitors, including competitive, non-competitive, and mechanism-based inhibitors (MBIs). CYP competitive inhibitors bind at the substrate-binding site, or bind elsewhere in or near the active site to block access or limit accessibility to the heme moiety (de Groot *et al.*, 2009). Non-competitive inhibitors can bind at a site distant from the substrate-binding site; they can change the confirmation of the CYP to prevent substrate binding or prevent the binding of co-enzymes to inhibit substrate metabolism (Coleman, 2010). A MBI is a substrate of an enzyme that is metabolized to a reactive intermediate that binds irreversibly to the enzyme rendering it inactive (Bertelsen *et al.*, 2003; Van *et al.*, 2006). Blocking an enzyme's metabolic activity can occasionally result in altered

drug efficacy or severe drug reactions, especially when multiple drugs that target the same CYP are co-administered.

Antidepressants such as selective serotonin reuptake inhibitors (e.g., fluoxetine) are substrates of CYP2D6 as well as potent inhibitors (**Table 2**) (Crewe *et al.*, 1992; Yamamoto *et al.*, 2003; Wang *et al.*, 2009). The co-administration of these antidepressants with neuroleptics, which are also CYP2D6 substrates, can result in adverse drug effects, such as neuroleptic induced extra-pyramidal side effects (EPS) (Spina *et al.*, 2001b; Saito *et al.*, 2005). The anti-arrhythmic drug quinidine is not a substrate of CYP2D6, but is able to selectively and potently inhibit CYP2D6 (Brosen *et al.*, 1987; Bertelsen *et al.*, 2003). The co-use of quinidine with antidepressants has been reported to increase adverse drug reaction (e.g., toxic delirium) and the co-use of quinidine with codeine has been reported to reduce codeine's analgesic response (Kathiramalainathan *et al.*, 2000; Trujillo & Nolan, 2000; Boyd, 2005; Ables & Nagubilli, 2010). Because of quinidines' selectivity and potent inhibition of CYP2D6, it is commonly used to identify the involvement of CYP2D6 in a metabolic pathway both *in vivo* and *in vitro* (Brosen *et al.*, 1987; Bertelsen *et al.*, 2003; McLaughlin *et al.*, 2005; Segura *et al.*, 2005).

Table 2: Examples of CYP2D6 inhibitors

Competitive Inhibitor	~K_i	References	Mechanism Based Inhibitor	~K_i	References
Citalopram	5.1 μM	(Crewe *et al.*, 1992)	Cimetidine	77 μM	(Madeira *et al.*, 2004)
Cocaine	1.1 μM	(Shen *et al.*, 2007)	Desethylamiodarone	4.5 μM	(Ohyama *et al.*, 2000)
Desipramine	1.7 μM	(Ball *et al.*, 1997)	MDMA	2 μM	(Van *et al.*, 2006)
Fluoxetine	1.6 μM	(Ball *et al.*, 1997)	Metoclopramide	4.7 μM	(Desta *et al.*, 2002)
Fluvoxamine	8 μM	(Ball *et al.*, 1997)	Paroxetine	4.9 μM	(Bertelsen *et al.*, 2003)
Haloperidol	7.2 μM	(Shin *et al.*, 1999)	Pimozide	0.8 μM	(Desta *et al.*, 1998)
Imipramine	29 μM	(Shin *et al.*, 1999)	Propranolol	1 μM	(Rowland *et al.*, 1994)
Methadone	3 μM	(Shin *et al.*, 2002)			
Metoprolol	21 μM §	(Komura & Iwaki, 2005a)			
Mirtazapine	41 μM	(Owen & Nemeroff, 1998)			
Propafenone	4 μM*	(McGinnity *et al.*, 2008)			
Quinidine	0.4 μM	(Brosen *et al.*, 1987)			
Thioridazine	1.4 μM	(Shin *et al.*, 2002)			
Timolol	7 μM§	(Volotinen *et al.*, 2007)			

The K_i values were obtained from human liver microsomes, human hepatocytes, or cDNA expressed CYP2D6 using the probe drugs dextromethorphan or debrisoquine. * the indicated value is the IC50, § the indicated value is the K_m for the substrate metabolism by CYP2D6, as their K_i values for CYP2D6 are currently undetermined.

1.4 CYP2D6 regulation

The regulation of CYP protein levels can occur at multiple stages, including transcription, translation, and post-translation (Aguiar *et al.*, 2005). Unlike most CYPs that are influenced by environmental factors, hepatic CYP2D6 displays constitutive expression during adulthood (Transon *et al.*, 1996; Stevens *et al.*, 2008). Though the regulation of hepatic CYP2D6 may be distinct from other CYPs, the CYP2D6 promoter region does have binding sites for both positively and negatively acting transcription factors (Yokomori *et al.*, 1995; Mizuno *et al.*, 2003b; Sakai *et al.*, 2009). Transcriptional regulation of hepatic CYP2D6 is largely controlled by the hepatocyte nuclear factor-4α (HNF-4α) transcription factor (Cairns *et al.*, 1996; Jover *et al.*, 1998; Corchero *et al.*, 2001). In the hepatic HepG2 cell line, transcription factors such as chicken ovalbumin upstream promoter transcription factor I (COUP-TFI) and nitric oxide (NO) were found to regulate CYP2D6 expression (Jover *et al.*, 1998; Hara & Adachi, 2002).

CYP2D levels are also affected by physiological changes. Interleukin-2 (released during inflammation) can induce hepatic CYP2D transcriptionally in rats (Kurokohchi *et al.*, 1992). During human pregnancy, there is a notable increase in CYP2D6 probe drug metabolism; however, the evidence of hepatic CYP2D6 protein induction during this period is unknown (Wadelius *et al.*, 1997). During human development, hepatic CYP2D6 protein expression and activity are very low in prenatal stages and do not correlate with *CYP2D6* genotype (Treluyer *et al.*, 1991; Stevens *et al.*, 2008). In the third trimester, CYP2D6 levels begin to increase to approximate 3 to 5% of CYP2D6 levels observed in an adult liver (Jacqz-Aigrain *et al.*, 1993). A dramatic increase occurs after birth until one year of age, independent of gestational age; this suggests that birth is a trigger for the increase in hepatic CYP2D6 expression (Treluyer *et al.*, 1991). There is no further change reported in hepatic CYP2D6

protein levels between the second year and adulthood (Transon *et al.*, 1996; Stevens *et al.*, 2008).

In contrast to other hepatic CYPs, CYP2D6 is relatively unaltered by common CYP inducers such as phenobarbital (Rae *et al.*, 2001; Edwards *et al.*, 2003). Although hepatic CYP2D6 is relatively uninducible and levels remain unaltered throughout most of adulthood, there is still remarkable variation in its expression and function. This variation is largely attributed to genetic variation.

1.5 *CYP2D6* genetic variation

The *CYP2D* gene cluster (**Figure 2**) is located on chromosome 22q13.1 with one functional gene (*CYP2D6)* and two pseudogenes (*CYP2D7P* and *CYP2D8P)* (Gonzalez *et al.*, 1988). The *CYP2D6* gene encompasses 9 exons and 8 introns with an open reading frame of 1383 base pairs coding for a 497 amino acid protein (Kimura *et al.*, 1989).

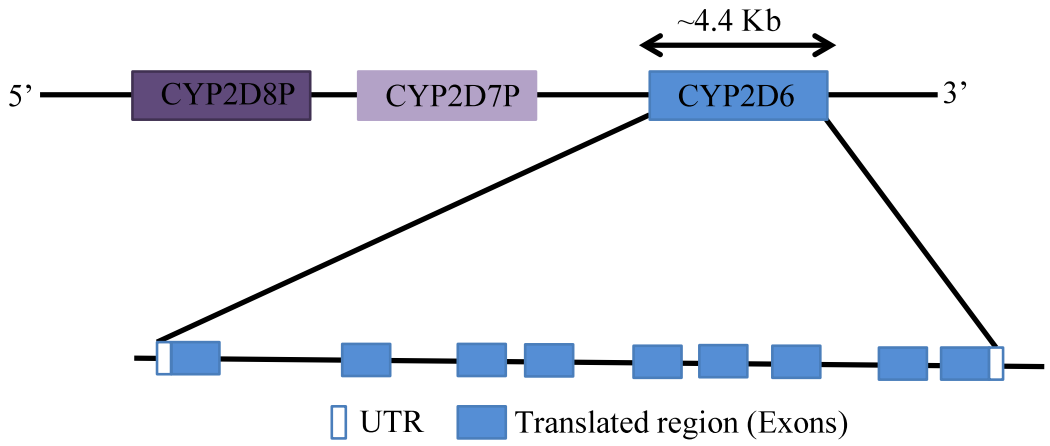


Figure 2: *CYP2D* gene cluster on chromosome 22q.13.1

To date, over 80 allelic variants in *CYP2D6* have been identified *(http://www.cypalleles.ki.se/cyp2d6.htm)*. The resulting allelic variants include full gene deletions, frameshifts, insertions, synonymous and non-synonymous changes, and copy number variants (resulting in up to 13 copies of CYP2D6) (Gaedigk *et al.*, 2008). These variant alleles can be categorized as null, reduced, normal, or increased function alleles (**Table 3**). This genetic variability contributes substantially to the interethnic and interindividual variability observed in CYP2D6 activity (e.g., as measured by dextromethorphan *O*-demethylation). The four main phenotypic categories are ultra-metabolizer (UM), extensive-metabolizer (EM), intermediate-metabolizer (IM), and poor-metabolizer (PM) (Gaedigk *et al.*, 2008). Individuals have traditionally been grouped into these four categories by *CYP2D6* genotype to predict CYP2D6 phenotype; however, considerable overlap in CYP2D6 phenotype (i.e., between EM and IM) remains among different genetic groups (Zanger *et al.*, 2004; Gaedigk *et al.*, 2008). This overlap is likely due to environmental factors or uncharacterized and/or unidentified genetic variability. In an attempt to better predict the CYP2D6 phenotype from the *CYP2D6* genotype, Gaedigk et al. (2008) proposed assigning an activity score to each allele. A functional score is given to each allele of 0 (null), 0.5 (reduced), 1 (normal), or 2 (increased), and the sum of both allele scores results in a predictive genotype based phenotype activity score (Gaedigk & Coetsee, 2008; Gaedigk *et al.*, 2008). **Table 3** lists functional and non-functional *CYP2D6* alleles in the four categories, along with the activity score, the change in CYP2D6 expression or function, and the allele frequency in Caucasians, Asians, and those of African descent.

Table 3: Common *CYP2D6* alleles and their frequencies in different ethnic populations

Activity	Allele	Change	Caucasians	Africans	Asians
Null (AS=0)	**3*	Frameshift, truncated protein (2549delA)	1-3.9%	0-0.6%	0.8-1%
	**4*	Splice defect, truncated protein (1846G>A)	12-23%	1.2-9.1%	0-2.8%
	**5*	Gene deletion	1.6-7.3%	0.6-6.9%	4.5-10%
	**6*	Frameshift (1707delT)	0.7-1.3%	0	-
Reduced (AS=0.5)	**9*	Lys deletion in exon 5, lower expression (2615_2617delAAG)	0-2.9%	0.69%	3.3%
	**10*	Protein stability (100C>T)	1.9-8%	3.1-8.6%	38-70%
	**17*	Decreased activity (1023C>T, 2850 C>T)	0.1-0.3%	10-34%	0.5%
	**29*	(2850C>T, 3183G>A, 4180G>C)	<0.1%	7-20%	-
	**41*	Splice defect, lower expression (2988G>A)	8-17%	15%	1.9-2.2%
Functional (AS=1)	**1*	Wild-type	33-76%	28-87%	21-42%
	**2*	(2850C>T, 4180G>C)	15-34%	11-78%	8-26%
Multiplication (AS≥2)	**1xN*	Increased protein expression	0.2-0.5%	3.3-22%	0.5%
	**2xN*	Increased protein expression	0.7-6.9%	1.6-54%	0-1%
	**4xN*	Truncated non-functional protein	0.1-0.2%	0.9-3%	-

AS, activity score (Zanger *et al.*, 2004; Gaedigk *et al.*, 2008).

1.5.A CYP2D pseudogenes

The pseudogene *CYP2D8P* is upstream to both *CYP2D7P* and *CYP2D6* (**Figure 2**). It contains multiple gene-disrupting insertions, deletions, and termination codons within its exons and does not transcribe into a functional CYP2D protein (Kimura *et al.*, 1989). *CYP2D7P* on the other hand, is identical in sequence to *CYP2D6* except for a T insertion in exon 1 (position 137) that causes a frameshift that disrupts the open reading frame (Zanger *et al.*, 2004). CYP2D7 mRNA has been isolated from the liver, and is expressed at similar levels to CYP2D6 mRNA (Endrizzi *et al.*, 2002). *CYP2D7* has the potential to encode a protein upon deletion of a single nucleotide that would restore the open reading frame. In an Asian Indian cohort, a 138delT was found in 6 out of 12 samples, and this deletion variant produced a functional CYP2D7 protein in expression studies (Pai *et al.*, 2004). However, other groups were unable to replicate these findings suggesting that it was unlikely that *CYP2D7* could generate a full-length functional protein (Gaedigk *et al.*, 2005b; Bhathena *et al.*, 2007; Zhang *et al.*, 2009).

1.5.B Interethnic variability CYP2D6

CYP2D6 polymorphisms resulting in null (i.e., PMs), reduced (i.e., IMs), or increased (i.e., UMs) activity vary greatly in frequency between different ethnicities (**Table 3**). This genetic variation can result in decreased or increased CYP2D6 enzymatic activity that can then affect an individual's response to ~30% of clinically prescribed drugs which are substrates of CYP2D6, and/or susceptibility to toxin-induced damage. Caucasians have the highest prevalence of PMs (5-10%) (Zanger *et al.*, 2004). This phenotype is primarily (70-90%) attributed to the *CYP2D6*4* allele because of the high frequency (20-25%) of this allele in this population (Zanger *et al.*, 2004; Neafsey *et al.*, 2009; Abraham *et al.*, 2010). The *CYP2D6*17* reduced-function allele occurs at the highest frequency (20-34%) in individuals of African

descent and this allele results in markedly lower CYP2D6 activity than wild-type (Gaedigk *et al.*, 2005a). In Asians, the most prevalent (37-70%) variant is the reduced-function allele *CYP2D6*10* and results in lower CYP2D6 activity than wild-type *CYP2D6* (Garcia-Barcelo *et al.*, 2000; Neafsey *et al.*, 2009). In contrast to null- or low-activity alleles, CYP2D6 copy number variants (e.g., gene duplications) resulting in a UM phenotype, occur at the highest frequency in North African (28-56%) and Middle Eastern (3-10%) populations. Ethiopians, Saudi Arabians, and Spaniards have the highest prevalence of the CYP2D6 UM phenotype representing ~29%, ~20% and ~10% of the total population, respectively (Agundez *et al.*, 1995; Dahl *et al.*, 1995; Aklillu *et al.*, 1996; McLellan *et al.*, 1997; Sachse *et al.*, 1997).

These ethnic differences in CYP2D6 may render some populations more likely to develop adverse drug reactions or altered drug effects at conventional doses (De Gregori *et al.*, 2010). For example, *CYP2D6* PMs on the neuroleptics haloperidol or risperidone show increased side effects, while CYP2D6 UMs taking the antidepressant imipramine show reduced drug effects (de Leon *et al.*, 2005; Ingelman-Sundberg *et al.*, 2007; Schenk *et al.*, 2008). These results can also be observed in UM, EM, or IM individuals who are co-treated with multiple substrates and/or inhibitors of CYP2D6 that consequently reduce, via inhibition, an individual's phenotype to PM (Coleman, 2010).

1.5.C CYP2D6 genetic associations to health risk

In addition to the *CYP2D6* genotype contributing to clinical drug outcomes, *CYP2D6* is also associated with some diseases and disorders. The *CYP2D6* genotype has been associated with cancer, epilepsy, suicide, the Lewy body variant of Alzheimer's disease, and PD (Borlak *et al.*, 1994; Cacabelos, 2008; Neafsey *et al.*, 2009; Ahlner *et al.*). There have been conflicting studies addressing the association of *CYP2D6* PMs and the risk for PD; however, a number of studies do find that *CYP2D6* PMs are overrepresented in PD cases (McCann *et al.*, 1997;

Coutts & Urichuk, 1999; Singh *et al.*, 2010). *CYP2D6* PMs are also at increased risk for neuroleptic-induced EPS (Fleeman *et al.*, 2010). As CYP2D6 can metabolize many neuroleptics, EPS in PMs likely results from the reduced ability of an individual to metabolize and clear the neuroleptics that induce EPS.

CYP2D6 can metabolize a number of neurotransmitters (discussed in **Section 2.2.B**), including dopamine (DA) and serotonin. It is therefore proposed that the *CYP2D6* genotype may also influence specific personality phenotypes (Hiroi *et al.*, 1998; Yu *et al.*, 2003b; Kirchheiner *et al.*, 2006; Penas *et al.*, 2009). Although the evidence is not entirely consistent, *CYP2D6* PMs more often present as being more anxious, more shy, less competitive, and more likely to avoid harm than EMs (Gan *et al.*, 2004; Roberts *et al.*, 2004; Gonzalez *et al.*, 2008).

1.6 CYP2D expression in different species

1.6.A CYP2D in rodents

CYP2D has been identified in a number of species including rat, mouse, and monkey (**Table 4**). Humans have one functional isoenzyme of CYP2D (i.e., CYP2D6), while rats have six different isoenzymes of CYP2D that display variation in substrate specificity, metabolism, and inhibition profiles (Strobl *et al.*, 1993; Hiroi *et al.*, 2002). An example of differences in inhibition between human and rat is the reduced potency of the selective inhibitor of human CYP2D6 quinidine to inhibit rat CYP2D whereas its stereoisomer, quinine, is highly selective and potent at CYP2D inhibition in rats (Tyndale *et al.*, 1999; Bertelsen *et al.*, 2003; Ai *et al.*, 2009). Each rat isoenzyme is differentially expressed in tissues with CYP2D1 and CYP2D2 being the most abundant isoforms in the liver (Wyss *et al.*, 1995; Haduch *et al.*, 2010). Notably, there are also differences in CYP2D expression between rat strains. For example, male Dark Agouti rats express much lower hepatic CYP2D2 than male Sprague Dawley rats. The difference in CYP2D expression is also sex specific. For example, while male Dark

Agouti rats express CYP2D2 in the liver, female Dark Agouti rats do not (Riedl *et al.*, 1999; Schulz-Utermoehl *et al.*, 1999). Female Dark Agouti rats are considered models for human CYP2D6 PMs because of their reduced ability to conduct CYP2D6-specific metabolism of substrates (e.g., debrisoquine, metoprolol, bufuralol) (Barham *et al.*, 1994; Schulz-Utermoehl *et al.*, 1999; Coleman *et al.*, 2000b).

Table 4: CYP2D isoenzyme amino acid identity to human CYP2D6

	Species	Isoenzyme	Identity	References
Primate	**Human**	**CYP2D6**	**100%**	(Igarashi *et al.*, 1997; Mankowski *et al.*, 1999; Hichiya *et al.*, 2004; Uno *et al.*, 2010; Uno *et al.*, 2011)
	Cynomolgus	CYP2D17 CYP2D44	93% 91%	
	Japanese	CYP2D29	96%	
	Marmoset	CYP2D19 CYP2D30	91% 95%	
	Rhesus	CYP2D42	91%	
Rodent	Rat	CYP2D1 CYP2D2 CYP2D3 CYP2D4 CYP2D5 CYP2D18	71% 71% 72% 77% 71% 77%	(Funae *et al.*, 2003; Yu & Haining, 2006; McLaughlin *et al.*, 2008)
	Mouse	Cyp2d9 Cyp2d10 Cyp2d11 Cyp2d12 Cyp2d13 Cyp2d22 Cyp2d24 Cyp2d26	69% 69% 67% 68% 65% 77% 69% 65%	

CYP2D isoenzymes in different species with % amino acid sequence identity compared to human CYP2D6.

Mice have nine different isoenzymes of *Cyp2d*, and Cyp2d22 is the most similar mouse Cyp2d isoenzyme to CYP2D6 (**Table 4**). However, Cyp2d22 does not share the same substrate specificity as CYP2D6 (Blume *et al.*, 2000; Yu & Haining, 2006). Purified Cyp2d22, and other mouse Cyp2ds, demonstrate impaired debrisoquine 4-hydroxylation and dextromethorphan *O*-

demethylation activity (Lofgren *et al.*, 2004; Yu *et al.*, 2004; Yu & Haining, 2006; McLaughlin *et al.*, 2008; Shen & Yu, 2009). As mice show little to no CYP2D6-like activity, a *CYP2D6* transgenic (*Tg*-CYP2D6) mouse line was created to better model human drug metabolism and drug interactions (Corchero *et al.*, 2001). These *Tg*-CYP2D6 mice provide a model that avoids the issues of species-, sex-, and isoenzyme-differences that have been observed in both CYP2D expression and activity in rats (Corchero *et al.*, 2001; Felmlee *et al.*, 2008; Shen & Yu, 2009). The *Tg*-CYP2D6 mice express their Cyp2d isoenzymes and human CYP2D6 in the liver, intestine, and kidney (Miksys *et al.*, 2005). *Tg*-CYP2D6 mice are able to 4-hydroxylate debrisoquine and *O*-demethylate dextromethorphan, pinoline, and 5-methoxy-*N*, *N*-dimethyltryptamine in contrast to wild-type mice that undergo these reactions at much lower levels (Yu *et al.*, 2003c; Yu & Haining, 2006).

1.6.B CYP2D in non-human primates

From a physiological perspective, monkeys are more similar to humans than are rodents. For example, there is a higher CYP amino acid sequence identity between monkeys and humans than between rodents and humans (**Table 4**) (Hichiya *et al.*, 2004; Iwasaki & Uno, 2009; Uno *et al.*, 2010). Monkey CYP2D can also metabolize CYP2D6 probe drugs efficiently in a similar manner to human CYP2D6 (i.e., bufuralol 1'-hydroxylation and dextromethorphan *O*-demethylation), and this metabolism can be inhibited by the selective CYP2D6 inhibitor quinidine (Hichiya *et al.*, 2004; Uno *et al.*, 2010). These similarities between monkeys and humans substantiate the use of monkeys as models of human drug metabolism.

2. Brain Cytochromes P450

2.1 Brain CYP expression

Cohn and colleagues reported the discovery of CYP expression in the brain in 1977 (Cohn *et al.*, 1977). To date, 41 of the 57 human CYP transcripts have been identified in the brain (Dauchy *et al.*, 2008; Dutheil *et al.*, 2009). However, only a small number of drug metabolizing enzymes (i.e., CYP1A, CYP1B, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A families) have been extensively studied in the brain at the transcript, protein, and/or activity level (Haining, 2007; Dauchy *et al.*, 2008; Dutheil *et al.*, 2009). CYP expression in the brain has been observed at the plasma membrane, endoplasmic reticulum, and mitochondria (Miksys *et al.*, 2000b; Howard *et al.*, 2003; Miksys *et al.*, 2003; Haining, 2007; Woodland *et al.*, 2008; Dutheil *et al.*, 2009). While this is unusual compared to liver, CYPs have also been identified in other tissues (i.e., lung, skin) in the cytoplasmic (CYP1A1) and the nuclear (CYP2E1) membrane, and they might have similar organelle expressions in the brain (Meyer *et al.*, 2002; Aguiar *et al.*, 2005; Meyer *et al.*, 2005; Seliskar & Rozman, 2007).

Brain CYPs display a great degree of variability in their regional and cellular expression (Dutheil *et al.*, 2009). Early reports suggested that brain CYP content is 1-5% of that found in the liver (Hedlund *et al.*, 1996; Gervasini *et al.*, 2004). However, because the brain is not a homogenous tissue and CYP expression varies across regions, this percentage range is unlikely to be true across all brain regions and for all CYPs. Within brain regions, CYPs display cell specific expression in pyramidal, Purkinje, granular, neuronal, astrocytic, and glial cells (Miksys *et al.*, 2000a; Howard *et al.*, 2003; Miksys *et al.*, 2003; Dutheil *et al.*, 2008). Although the overall CYP content is lower in the brain cellular expression of CYPs (e.g., CYP2B in pyramidal neurons) can be equal to, or even higher than, levels found in hepatocytes (Miksys *et al.*, 2000a). This highly localized CYP expression is believed to create

microenvironments in the brain that likely contribute to localized drug metabolism (Britto & Wedlund, 1992; Miksys & Tyndale, 2009).

Another part of the brain that expresses CYPs is the blood brain barrier (BBB). The BBB is a neurovascular unit made up of endothelial cells with tight junctions, pericytes, and astrocytes (Alam *et al.*, 2010). The BBB is the first line of defence against physical and chemical intrusion, and protects the brain from harmful chemicals in the blood (Miller, 2010). The predominant CYP mRNA transcripts identified at the BBB of humans are CYP1B1 and CYP2U1, and, at lower levels of expression, CYP2E1 and CYP2D6 (Dauchy *et al.*, 2008). CYP2B6 expression has been observed at the BBB of rodents and in human astrocytes (including in their end-feet, which sheath the endothelial cells of the BBB) (Miksys *et al.*, 2000a; Miksys *et al.*, 2003). Recently, the expression of CYP3A protein was reported in endothelial cells in the human brain (Ghosh *et al.*, 2010; Ghosh *et al.*, 2011). The localization of CYPs at the BBB makes them ideally situated to protect the brain against drugs and environmental toxins.

2.1.A Brain CYP activity

The functional importance of CYPs in the brain may be to protect against toxicity from exogenous substrates (e.g., drugs, toxins) and/or to metabolize endogenous neurochemicals (e.g., neurosteroids, biogenic amines) (Haining, 2007). Select CYPs can metabolize arachidonic acid, pregnenolone, estradiol, androstenedione, testosterone, 5-hydroxytryptamine, and melatonin (Rifkind *et al.*, 1995; Doostzadeh & Morfin, 1997; Rosenbrock *et al.*, 1999; Ohe *et al.*, 2000; Wang *et al.*, 2000; Fradette *et al.*, 2004; Ma *et al.*, 2005). Brain CYPs are enzymatically active *in vitro* and, of those assessed, have similar substrate affinities to their respective hepatic CYP isoforms (Bhamre *et al.*, 1993; Ghersi-Egea *et al.*, 1993; Tyndale *et al.*, 1999; Bhagwat *et al.*, 2000; Voirol *et al.*, 2000).

Brain CYPs are likely active *in situ*, as expression of the important co-enzyme CYP-reductase has been detected in the brain (Haglund *et al.*, 1984; Ghersi-Egea *et al.*, 1989; Bergh & Strobel, 1996; Riedl *et al.*, 1996; Conroy *et al.*, 2010). Our work on rats involving intra-cerebral delivery of a CYP2B radiolabeled MBI (^{3}H-8-methoxypsoralen) demonstrated that constitutively expressed and nicotine induced, CYP2B is active *in situ* (Miksys & Tyndale, 2009). By inhibiting brain but not hepatic CYP2B with the MBIs C8-xanthate and 8-methoxypsoralen, we have shown significant changes in the drug response of propofol (which is inactivated by CYP2B) wherein propofol-induced sleep time was increased (Kokhar & Tyndale, 2010). Although brain metabolism may not greatly influence overall xenobiotic clearance, these results indicate differences in brain CYP levels can influence localized drug metabolism and thereby drug response.

2.1.B Brain CYP regulation

CYPs in the brain display CYP-, organ-, region-, cell type-, and inducer-specific modes of induction (Miksys & Tyndale, 2002; Miksys & Tyndale, 2004). *In vivo*, nicotine and ethanol can induce brain CYPs, and these CYPs can display organ-specific and CYP-specific induction patterns. In monkeys, for example, nicotine induces CYP2B in the brain but not in the liver and CYP2E1 in both the liver and the brain (Miksys *et al.*, 2000a; Joshi & Tyndale, 2006a; Lee *et al.*, 2006a). In rats, ethanol induces CYP2E1 in the brain and the liver, but CYP2B in the liver and not in the brain (Warner & Gustafsson, 1994; Howard *et al.*, 2003; Schoedel & Tyndale, 2003). In human studies, both cigarette smoking and alcohol use are associated with altered human brain CYP expression. Cigarette smokers have higher levels of CYP2B6 and CYP2E1 in select brain regions (e.g., striatum) than non-smokers, and alcoholics have higher levels of brain CYP2B6 and CYP2E1 in select brain regions than do non-alcoholics (Howard *et al.*, 2003; Miksys *et al.*, 2003).

2.2 Brain CYP2D6

2.2.A CYP2D6 CNS expression

The expression of CYP2D in the brain has been identified in rat, mouse, dog, monkey, and human (Fonne-Pfister *et al.*, 1987; Niznik *et al.*, 1990; Tyndale *et al.*, 1991; Tyndale *et al.*, 1999; Siegle *et al.*, 2001; Miksys *et al.*, 2005). Of the 6 rat CYP2D isoenzymes, CYP2D4 is predominantly expressed in the brain and CYP2D18 is thought to be exclusively expressed in the brain (Coleman *et al.*, 2000b). In the mouse, only Cyp2d22, the structural ortholog of CYP2D6, has been detected in the brain (Singh *et al.*, 2009). Of the 24 CYP transcripts that have been identified in the brain, CYP2D6 is one of the more prominent drug metabolizing CYPs that is expressed in most human brain regions (Dutheil *et al.*, 2009). CYP2D6 is expressed in the human neocortex, caudate, putamen, globus pallidus, nucleus accumbens, hippocampus, hypothalamus, thalamus, substantia nigra (SN), cerebellum, and medulla oblongata (Gilham *et al.*, 1997; McFayden *et al.*, 1998; Siegle *et al.*, 2001; Miksys *et al.*, 2002). CYP2D6 protein expression is highest in the caudate, putamen, cortex, and cerebellum, and lowest in the hippocampus and globus pallidus (Miksys *et al.*, 2002).

Notably, CYP2D6 protein expression is also cell type-specific. For example, intense immunoreactivity of CYP2D6 is found in pigmented neurons of the SN, pyramidal cells of the hippocampus and frontal cortex, and Purkinje cells of the cerebellum, glial cells, astrocytes, and endothelial cells at the BBB (Gilham *et al.*, 1997; Siegle *et al.*, 2001; Miksys *et al.*, 2002; Dauchy *et al.*, 2009; Dutheil *et al.*, 2010). This corresponds with rat brain CYP2D which is expressed in the same cell types including the pigmented neurons of the SN, and rat CYP2D has been demonstrated to co-localize with tyrosine hydroxylase a marker for dopaminergic neuronal cells (Michels & Marzuk, 1993a; b; Watts *et al.*, 1998; Riedl *et al.*, 1999). The expression of CYP2D in dopaminergic neurons is further supported by the decrease in CYP2D

detection upon lesioning of the nigrostriatal tract with the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) (Watts *et al.*, 1998). This expression is important, as dopaminergic neurons are lost during PD, indicating that CYP2D is ideally situated to inactivate neurotoxin-substrates that may elicit dopamine-cell specific damage.

2.2.B CYP2D6 CNS function

CYP2D activity in brain membranes has been measured using 1'-hydroxylation of bufuralol, *O*-demethylation of codeine to morphine, and *O*-demethylation of dextromethorphan in rats and sparteine metabolism in dogs (Chen *et al.*, 1990; Tyndale *et al.*, 1991; Lin *et al.*, 1992; Jolivalt *et al.*, 1995; Coleman *et al.*, 2000b; Voirol *et al.*, 2000). Brain CYP2D substrate affinities (K_m) are similar to those observed with hepatic CYP2D; however, the substrate turnover (V_{max}) is much lower as a result of lower CYP expression (Tyndale *et al.*, 1991; Coleman *et al.*, 2000b). Rat CYP2D in different brain regions can metabolize dextromethorphan to varying degrees (Tyndale *et al.*, 1999). The rat cerebellum demonstrated the highest CYP2D protein expression and greatest dextromethorphan metabolism. Notably, CYP2D activity between brain regions correlates with both CYP2D mRNA and protein expression (Tyndale *et al.*, 1999).

Chen et al., (1990) demonstrated that rat whole brain microsomes and microvessels were able to metabolize codeine to morphine, a CYP2D mediated pathway. Thirty minutes after administering codeine (20 mg/kg, i.p.) they observed significantly more codeine in the brain compared to the plasma. Further, 30 minutes after the administration of codeine (i.p.) they observed significantly higher levels of morphine in the brain than the plasma, compared to the delivery of 1 mg/kg morphine (i.p.); a dose that resulted in equivalent plasma levels of morphine to 30 minutes after 20 mg/kg (i.p.) of codeine administration. As codeine is metabolized to morphine primarily by CYP2D6, the higher brain morphine levels in the first 30

minutes after codeine administration were attributed to the localized activation of codeine by brain CYP2D (Chen *et al.*, 1990). Additionally, measuring MPTP metabolites in rat brain post-MPTP brain infusions showed localized inactivation of MPTP to PTP in the striatum, a metabolite largely produced by CYP2D (Vaglini *et al.*, 2004). Together, these studies support *in situ* CYP2D metabolism in the brain.

The relevance of CYP2D6 expression in the brain is multifaceted. Not only does CYP2D6 1) metabolize a large number of CNS-acting drugs and 2) inactivate neurotoxins, it can also 3) metabolize endogenous CNS compounds (**Table 5**), such as biogenic amines and neurosteroids (Hiroi *et al.*, 2001; Yu *et al.*, 2003c). For example, cDNA expressed CYP2D6 and *Tg*-CYP2D6 mice can *O*-demethylate 5-methoxytryptamine to serotonin (Yu *et al.*, 2003c). CYP2D6 expressed in yeast cells can also generate DA from *p*-tyramine and *m*-tyramine (Hiroi *et al.*, 2001). Furthermore, rat brain membranes isolated from different brain regions can variably metabolize *p*- and *m*-tyramine to DA, with the highest DA formation observed in the SN and cerebellum (Bromek *et al.*, 2010). The fact that CYP2D6 can metabolize DA and other biogenic amines (See **Table 5),** supports the premise that variation in brain CYP2D6 may affect neurobehaviour (discussed in **Section 1.5.C**) (Hiroi *et al.*, 2001; Yu *et al.*, 2003c). Overall, these findings suggest an important functional role for brain CYP2D6.

Table 5: Examples of endogenous CNS substrates of CYP2D

Amines/Neurotransmitters	
Substrate	**Product**
p/m-Tyramine	Dopamine
5-Methoxytryptamine	5-Hydoxytryptamine
Octopamine	Norepinephrine
Synephrine	Epinephrine
5-Methoxy-*N*-*N*-dimethyltryptamine	Bufotenine
4-Methoxyphenethylamine	Tyramine
3-Methoxyphenethylamine	3-Hydroxyphenethylamine
Pinoline	6-Hydroxytetrahydro-β-carboline
Harmaline	Harmalol
Harmine	Harmol
Harman	6-Hydroxy-harman
Norharman	6-Hydroxy-norharman

Steroids[§]	
Substrate	**Reaction**
Estradiol	2-Hydroxylation
Progesterone	6β–, 16α–, 2β– or 21-Hydroxylation
Allopregnanolone	21-Hydroxylation
Testosterone	2β–, 6β– or 17-Hydroxylation
Estrone	2-Hydroxylation

Fatty acid /Amide[§]	
Substrate	**Reaction**
Arachidonic acid*	*w*-Hydroxylation
Anandamide	*w*-, *w*-1, *w*-2 or *w*-3 Hydroxylation

*The metabolism of arachidonic acid was only observed with cDNA expressed CYP2D18 and has not been observed with human liver microsomes or cDNA expressed CYP2D6 (Rifkind *et al.*, 1995; Thompson *et al.*, 2000; Snider *et al.*). References for amine metabolism are (Hiroi *et al.*, 1998; Thompson *et al.*, 2000; Miller *et al.*, 2001; Yu *et al.*, 2003a; Yu *et al.*, 2003c; Yu *et al.*, 2003d; Herraiz *et al.*, 2008; Snider *et al.*, 2008; Jiang *et al.*, 2009; Shen *et al.*, 2011). [§]CYP2D6 can catalyze hydroxylation at multiple positions on steroids and anandamide; hence the specific reactions are listed as opposed to metabolites (Hiroi *et al.*, 2001; Lee *et al.*, 2003; Kishimoto *et al.*, 2004; Niwa *et al.*, 2008). For a good review, see Wang et al., (2009).

2.2.C Brain CYP2D6 regulation

Unlike CYP2D6 in the liver, which is relatively uninducible, brain CYP2D can be induced by multiple CNS-acting drugs in a drug-specific and brain region-specific manner (Rae *et al.*, 2001; Edwards *et al.*, 2003). For example, the neuroleptic drug clozapine (1 mg/kg, gavage, 3 weeks) increased cellular staining for rat brain CYP2D in neurons of the SN, the ventral tegmental area, the granular neurons of the olfactory bulb, and in the Purkinje and granular neurons of the cerebellum (Hedlund *et al.*, 1996). There was no notable change in CYP2D mRNA suggesting the induction of brain CYP2D likely occurred through post-transcriptional changes. Additionally, no change in CYP2D was observed in the liver. Fluoxetine (5 mg/kg, i.p., 2 weeks, b.i.d.) increased CYP2D protein by ~1.5-fold and CYP2D activity by ~1.3-fold in rat cerebellum (Haduch *et al.*, 2010). Similarly, thioridazine (10 mg/kg, i.p., 2 weeks, b.i.d.) increased CYP2D protein by ~2-fold in the SN and ~1.5-fold in rat cerebellum along with a ~1.25-fold increase in CYP2D activity in both regions (Haduch *et al.*, 2010). Treatment with toluene (1 mg/kg, i.p., 3 days) increased mRNA, protein, and the activity of CYP2D4 in rat brain (Mizuno *et al.*, 2003a).

Chronic treatment with nicotine (1 mg/kg for 7 days) induced rat brain CYP2D in a region-specific and a cell-specific manner without changing the levels of CYP2D in the liver (Yue *et al.*, 2008). The induction was observed in the cerebellum, hippocampus, and striatum. CYP2D induction persisted for up to 8 hours post-injection, returning to baseline by 12 hours. Using slot blot, no change in CYP2D mRNA was detected in the brain suggesting that induction is post-transcriptional (Yue *et al.*, 2008).

We have found that human alcoholics have higher brain CYP2D6 levels compared to non-alcoholics (Miksys *et al.*, 2002). In a rat study, a single dose of ethanol (200 μL, i.p.) induced CYP2D in rat brain (Warner & Gustafsson, 1994).

Endogenous compounds such as steroids can also regulate CYP2D. Sex steroids, such as estrogen and testosterone, can alter CYP2D mRNA in select brain regions of ovariectomized rats (Bergh & Strobel, 1996). Notably, treatment with a combination of testosterone and estrogen suppressed brain CYP2D induction when compared to testosterone alone. This suggests that estrogen may have repressive effects on CYP2D levels in the brain.

Overall, the ability of compounds to induce brain CYP2D6 not only adds another level of variability in substrate metabolism, but also suggests the possibility of altered susceptibility to drug toxicity, therapeutic response to drugs, drug abuse, neurotoxicity, and/or neurological disorders such as PD.

3. Parkinson's Disease

PD is a progressive neurodegenerative disorder that presents with asymmetrical symptoms as a result of severe damage to the SN. This damage eventually progresses and affects both sides of the brain (Factor & Weiner, 2008). Cardinal symptoms are resting tremors, rigidity, bradykinesia, postural instability, and gait disturbance; any two must be observed to diagnose PD (Factor & Weiner, 2008). There are also a number of non-motor symptoms that occur in both early and late stages of PD, including dementia, depression, visual hallucinations, autonomic dysfunction, anxiety, impulsivity, and apathy (Buck *et al.*, 2010). A number of pre-motor symptoms are currently being studied as risk factors for PD, including olfactory dysfunction, sleep abnormalities, constipation, depression, and alteration in pain (Obeso *et al.*, 2010).

PD is the second most prevalent neurodegenerative disease, affecting 1–2% of individuals older than 60 years of age (Dawson *et al.*, 2010). On average, PD is diagnosed between 60 and 65 years of age (late-onset PD); however, PD cases as young as 25 years of age (early-onset PD) have also been reported (Lees, 2010). The disease is more common in males than females with a relative risk of 1.5 in males (**Figure 3**) (Taylor *et al.*, 2007; Muangpaisan *et al.*, 2009). The incidence for PD increases with age and ranges from 1.5 to 20 per 100,000 people per year across world regions (Twelves *et al.*, 2003; Muangpaisan *et al.*, 2009). Some current studies report little to no change in incidence over the past twenty years (Muangpaisan *et al.*, 2009; Lix *et al.*, 2010). The prevalence of PD varies across the globe (**Figure 4**); the crude prevalence by location ranges from 27 per 100,000 people in Saudi Arabia to 439 per 100,000 in Australia (al Rajeh *et al.*, 1993; Chan *et al.*, 2005).

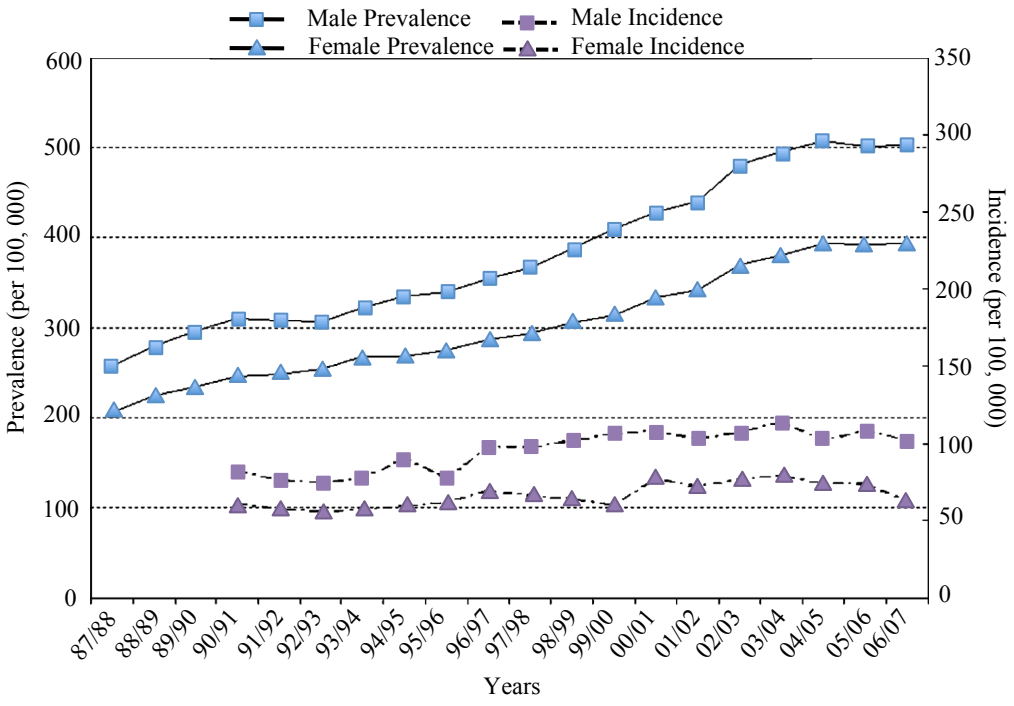


Figure 3: Prevalence and incidence rates of Parkinson's disease (Canada)
This figure is adapted from Lix et al., (2010) by permission from the copyright holder.

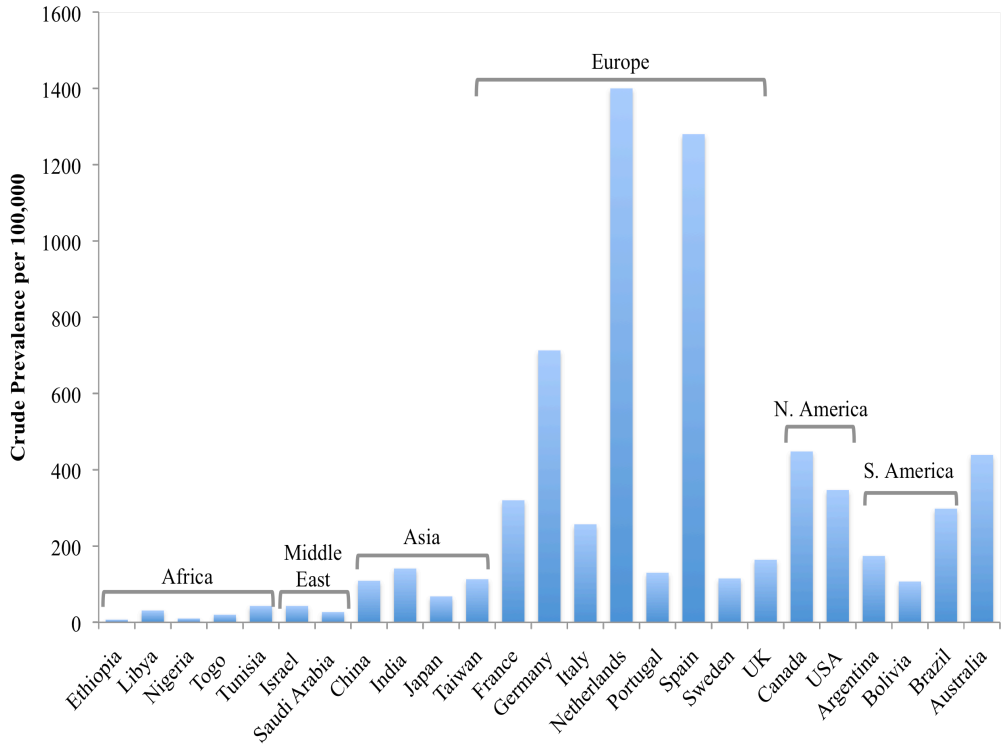


Figure 4: Crude prevalence of Parkinson's disease across different world populations
The prevalence of PD in different populations were graphed from the following references: Africa (Okubadejo *et al.*, 2006); Middle East (al Rajeh *et al.*, 1993; Masalha *et al.*); Asia, Australia, and South America (Muangpaisan *et al.*, 2009); Europe (von Campenhausen *et al.*, 2005); and North America (Schoenberg *et al.*, 1985; Lix *et al.*, 2010)

3.1 Pathogenesis

The neuropathology of PD is characterized by the initial loss of dopaminergic neurons in the SN that project to the striatum (Fahn, 2010). However, the degree of neuronal loss is more pronounced in the striatum than in the SN pars compacta (Bernheimer *et al.*, 1973). Symptoms occur when approximately 60% of dopaminergic neurons are lost in the SN pars compacta. The greatest loss occurs in the ventrolateral tier, followed by loss in the medial ventral tier, and the dorsal tier (Lang & Lozano, 1998). In the striatum it is primarily the dopaminergic neurons projecting to the putamen that are affected, with predominant loss in the dorsal lateral putamen (Taylor *et al.*, 2007). Neurons lost during PD accumulate cytoplasmic

inclusion bodies of misfolded α-synuclein (α-syn). These inclusion bodies, known as Lewy bodies, are a hallmark of PD and are observed in ~90% of cases (Lang & Lozano, 1998).

The progression of PD is staged into six neuropathological stages known as Braak stages. These stages are based on the continual development of interneuronal lesions and the distribution of Lewy bodies and Lewy neurites in select areas of the brain. Braak stages consist of two presymptomatic, two symptomatic and two end stages (Braak *et al.*, 2004). In the end stages, when inclusion bodies and lesions invade cortical regions, a large portion of patients (~40%) develop dementia and other psychological symptoms (e.g., hallucinations) (Fahn, 2010). The rate of the disease's progression varies between cases (i.e., slow or rapid), and, if not interrupted by death, the disease eventually progresses to very severe symptoms (Pahwa, 2003).

3.1.A Mechanism of cell death

The neuronal loss observed in PD involves both programmed (apoptosis) and lysosome-mediated (autophagy) cell death (Hartmann *et al.*, 2000; Burbulla *et al.*, 2010). Oxidative stress is thought to be the main mechanism leading to cell death in PD, as a result of lipid, protein, and DNA oxidation. These oxidative reactions occur primarily with reactive oxygen species (ROS), including peroxides, quinones, hydroxyl radicals, heavy metals, and nitric oxide (NO) (Migliore & Coppede, 2009). In fact, increased levels of iron, manganese, and hydroxyl free radicals have been reported in post-mortem PD brain tissue, supporting a role for oxidative stress damage in PD (Jenner *et al.*, 1992; Asanuma *et al.*, 2004). Some endogenous sources of ROS are DA auto-oxidation, DA metabolism, and NADPH-oxidase in microglia and mitochondria (Barzilai *et al.*, 2001; Tieu *et al.*, 2003; Patten *et al.*, 2010).

Oxidative stress results from both excessive production of ROS and limited antioxidant defences. Antioxidant defence enzymes, such as glutathione, superoxide dismutases,

glutathione peroxidase, and catalase, convert reactive species to less reactive compounds (Ballatori *et al.*, 2009). PD post-mortem brain tissue has ~40% less glutathione peroxidase content in the SN than non-PD brain tissue, and surviving neurons show a significant loss of glutathione peroxidase (Sian *et al.*, 1994; Pearce *et al.*, 1997). Depletion of glutathione peroxidase can enhance cell death especially when other cellular functions are damaged, as is the case in mitochondrial dysfunction (Ballatori *et al.*, 2009).

Mitochondria maintain cellular function by generating energy for the cell; therefore, impairment in this function can contribute to cell death (Burbulla *et al.*, 2010). Biochemical and histological studies in post-mortem PD SN pars compacta show reduced complex I activity, a component of the mitochondrial respiratory chain (Schapira *et al.*, 1990). The role of mitochondrial dysfunction in cell death is further supported by the ability of PD-causing neurotoxins (e.g., rotenone, MPTP) to inhibit complex I (Watabe & Nakaki, 2008). Complex I inhibition interrupts mitochondrial energy metabolism and increases ROS formation, leading to cell death (Ramachandiran *et al.*, 2007). In PD cases, proteins involved in maintaining mitochondrial homeostasis and integrity, such as Parkin and PTEN-induced kinase (PINK), show impaired transport to and into the mitochondria (Burbulla *et al.*, 2010). Loss of function variants in these proteins, as observed in PD cases, results in mitochondrial dysfunction *in vitro* and *in vivo* (Rothfuss *et al.*, 2009; Weihofen *et al.*, 2009). Together these data support the involvement of mitochondria in the mechanism of cell death in PD.

In addition to oxidative stress and mitochondrial dysfunction, immune response and excitotoxicity, are also believed to contribute to cell death in PD (Factor & Weiner, 2008). Immune response has been implicated as a player in PD pathogenesis because activated microglia, increased cytokine expression, and upregulation of inflammatory factors have been detected in the striatum and cerebral spinal fluid of PD patients (Olanow, 2007). Excitotoxicity

has been implicated in PD, since excessive *N*-methyl-*D*-aspartate receptor stimulation by glutamate can induce cellular toxicity (Factor & Weiner, 2008). Furthermore, the use of *N*-methyl-*D*-aspartate receptor antagonists can reduce neurotoxin-induced neuronal damage *in vivo* (Factor & Weiner, 2008). The over stimulation of the *N*-methyl-*D*-aspartate receptor leads to increased Ca^{++}, NO synthesis and oxidative stress to promote cellular damage and subsequently cell death (Koutsilieri & Riederer, 2007).

3.1.B Selective degeneration of dopaminergic neurons

Analysis of post-mortem PD tissues indicates the SN experiences increased levels of protein, lipid, and DNA oxidation, likely resulting from high degrees of oxidative stress (Dexter *et al.*, 1989; Alam *et al.*, 1997; Floor & Wetzel, 1998). The cause of select dopaminergic neuronal loss in the SN is still unclear. DA has been long viewed as a culprit for this selective loss of neurons, especially as presynaptic nerve terminals in the striatum are most sensitive to damage (Jenner & Olanow, 1996). DA is rapidly metabolized by monoamine oxidase generating reactive bi-products (e.g., H_2O_2), and by auto-oxidation to quinones and superoxide anions (Gonzalez-Hernandez *et al.*, 2010). Both enzymatic and non-enzymatic catabolism of DA are important sources of ROS and have been implicated in dopaminergic cell degeneration. High DA content and turnover around these synapses may, therefore, increase oxidative stress and produce severe damage (Parent & Parent, 2010).

Another potential contributor to SN dopaminergic cell loss is neuromelanin, a pigmented polymer composed of melanin, proteins, lipids, and trace metals (Double *et al.*, 2000). Neuromelanin is found at very high concentrations in the SN giving this region its characteristic dark pigmentation (Sian-Hulsmann *et al.*, 2010). In the SN, DA neurons that contain neuromelanin are preferentially lost during PD, which suggests that neuromelanin may be involved in PD pathogenesis (Fasano *et al.*, 2006; Gonzalez-Hernandez *et al.*, 2010).

Neuromelanin is toxic and can activate mitochondrial apoptosis signalling *in vitro* (Li *et al.*, 2005; Naoi *et al.*, 2008). *In vitro,* cell death caused by neuromelanin is further increased in the presence of iron (a contributor to oxidative stress), and this enhancement is abolished by iron chelators (Das *et al.*, 1978; Zecca *et al.*, 2004). It has been noted that PD cases have higher iron content in the SN compared to controls, and this indicates that both high levels of neuromelanin and iron may contribute to increased susceptibility of SN neurons to oxidative damage (Hirsch *et al.*, 1991; Barzilai *et al.*, 2001).

Some additional factors believed to enhance the sensitivity of dopaminergic neurons include changes in calcium homeostasis, the size of DA neurons, a high degree of dopaminergic connections, and a high rate of oxygen consumption (Chinta & Andersen, 2005; Obeso *et al.*, 2010; Surmeier *et al.*, 2010). Although the exact factors responsible for the degeneration of specific neuronal groups is still uncertain, intrinsic factors (i.e., L-type calcium channels) seem to be preferentially involved in the initiation of this damage.

3.2 Etiology

PD is divided into two major forms, familial PD that represents ~10% of cases and sporadic PD that represents ~90% of cases (Vance *et al.*, 2010). Familial PD cases have provided great insight into mechanisms associated with PD including the discovery of leading gene candidates involved in the etiology of PD such as *α-synuclein* (*SNCA)*, *Parkin*, *PINK1,* and *Leucine Rich Repeat Kinase 2 (LRRK2)* (Urdinguio *et al.*, 2009). Unlike familial PD, which is primarily attributed to genetic risk, sporadic PD is thought to occur from the combination of genetic predisposition and environmental exposure to toxins.

The primary environmental compounds associated with increased PD risk are multiple classes of pesticides (e.g., insecticides, herbicides, fungicides) (Hatcher *et al.*, 2008; Horowitz & Greenamyre). Most epidemiological studies, including meta-analyses, have consistently

shown that pesticide exposure increases risk for PD. Paraquat and rotenone are of particular interest, as they increase risk for PD and elicit PD-like pathology in animal models (Sherer *et al.*, 2003; Kamel *et al.*, 2007; Dhillon *et al.*, 2008; Hatcher *et al.*, 2008; Ritz *et al.*, 2009). The odds ratio of risk for PD upon pesticide exposure varies between countries, ranging from 1.7 (95% CI=1.1, 2.8) to 7 (95% CI=1.61, 63.46) (Priyadarshi *et al.*, 2000; Hancock *et al.*, 2008; Ritz *et al.*, 2009). Pesticide exposure also shows a dose-dependent effect in duration of usage and increased risk for PD (Le Couteur *et al.*, 1999; Petrovitch *et al.*, 2002; Brown *et al.*, 2006). Living in a rural environment and drinking well water, which may be related to pesticide exposure, can also increase the risk for PD (Gorell *et al.*, 1998; Factor & Weiner, 2008). Other environmental risk factors include exposure to heavy metals (e.g., iron, copper) and solvents, or working in welding or mining operations (Hatcher *et al.*, 2008; Sanyal *et al.*, 2010; Sriram *et al.*, 2010).

3.2.A Genes implicated in PD

Many genes contribute to inherited PD and increase risk for sporadic PD (West *et al.*, 2002; Valente *et al.*, 2004b). Of the genes discovered, five have been shown to produce pathological features that resemble PD in genetic animal models: *SNCA*, *Parkin, DJ1, PINK1,* and *LRRK2* (Valente *et al.*, 2004a; Tan & Skipper, 2007). Genetic variants in the *SNCA* gene that result in amplified α-syn protein formation (i.e., duplication or triplication), or α-syn misfolding and/or aggregation, cause increased Lewy body formation and the development of a severe PD phenotype (Polymeropoulos *et al.*, 1997; Singleton *et al.*, 2003; Wang *et al.*, 2006). Toxin exposure and oxidative stress can also upregulate *SNCA* resulting in enhanced aggregation and the promotion of α-syn binding to DA, metals, certain proteins, and lipids (Giasson *et al.*, 2002; Lee & Trojanowski, 2006). The mechanism behind α-syn aggregation

induced cell death is undetermined, but the formation of inclusion bodies is thought to interfere with neuronal transport and the regulation of genes (Horowitz & Greenamyre, 2010).

Variants in *Parkin*, a multifunctional E3 ubiquitin protein ligase, and *PINK1* are prevalent in familial PD (Burbulla *et al.*, 2010). Both Parkin and PINK1 are involved in maintaining mitochondrial integrity and function (Kim *et al.*, 2008; Horowitz & Greenamyre, 2010). Variants that result in a non-functional Parkin protein consequently lead to impaired mitochondrial integrity (Park *et al.*, 2006). Upon mitochondrial dysfunction, both Parkin and PINK1 are recruited from the cytosol to promote autophagic clearance from the cell and thus may participate in a protective response (Burbulla *et al.*, 2010).

Gain-of-function variants in *LRRK2* occur in 5–10% of familial PD cases and in 1–5% of sporadic PD cases (Wood-Kaczmar *et al.*, 2006; Hindle, 2010). Unlike the above-mentioned genes, patients with *LRRK2* variants exhibit late-onset PD which resemble sporadic PD cases (Cookson, 2010). How LRRK2 is involved in cell death is still unclear, but it is known that it can interact with Parkin and bind to the outer-membrane of the mitochondria (Olanow, 2007; Dawson *et al.*, 2010).

3.2.B Neurotoxins implicated in Parkinson's disease

In addition to genetic factors, environmental and endogenous neurotoxins are also implicated in PD etiology. The discovery of MPTP, a Parkinsonian syndrome causing neurotoxin, strongly suggested the role of exogenous toxins in PD pathogenesis (Langston & Ballard, 1983). This finding prompted investigations into a link between environmental toxins (e.g., pesticides) and PD, leading to the identification of rotenone and paraquat as possible PD-neurotoxin candidates (Ramachandiran *et al.*, 2007; Meredith *et al.*, 2008; Costello *et al.*, 2009; Horowitz & Greenamyre, 2010). Rotenone is an insecticide that selectively causes the loss of DA neurons in the SN *in vitro* and *in vivo* (Sherer *et al.*, 2003; Jin *et al.*, 2007). It is a potent

inhibitor of mitochondrial complex I. This inhibition results in mitochondrial dysfunction, increased oxidative stress, protein aggregation, and, consequently, cell death (Jin *et al.*, 2007). The herbicide paraquat can induce nigrostriatal degeneration in rodents and is toxic in *in vitro* neuronal models (Liou *et al.*, 1996; Brooks *et al.*, 1999). Unlike rotenone, paraquat does not inhibit complex I, but instead produces free radicals, inhibits proteosomal activity, and promotes protein aggregation (Wang *et al.*, 2006; Lee *et al.*, 2008a). Notably, paraquat structurally resembles MPP^+, the potent neurotoxic metabolite of MPTP (**Figure 5A**), but is not transported into the dopaminergic cells by the DAT like MPP^+ (Wang *et al.*, 2006; Lee *et al.*, 2008a). These findings suggested that structurally related exogenous or endogenous compounds may be responsible for neuronal damage in PD.

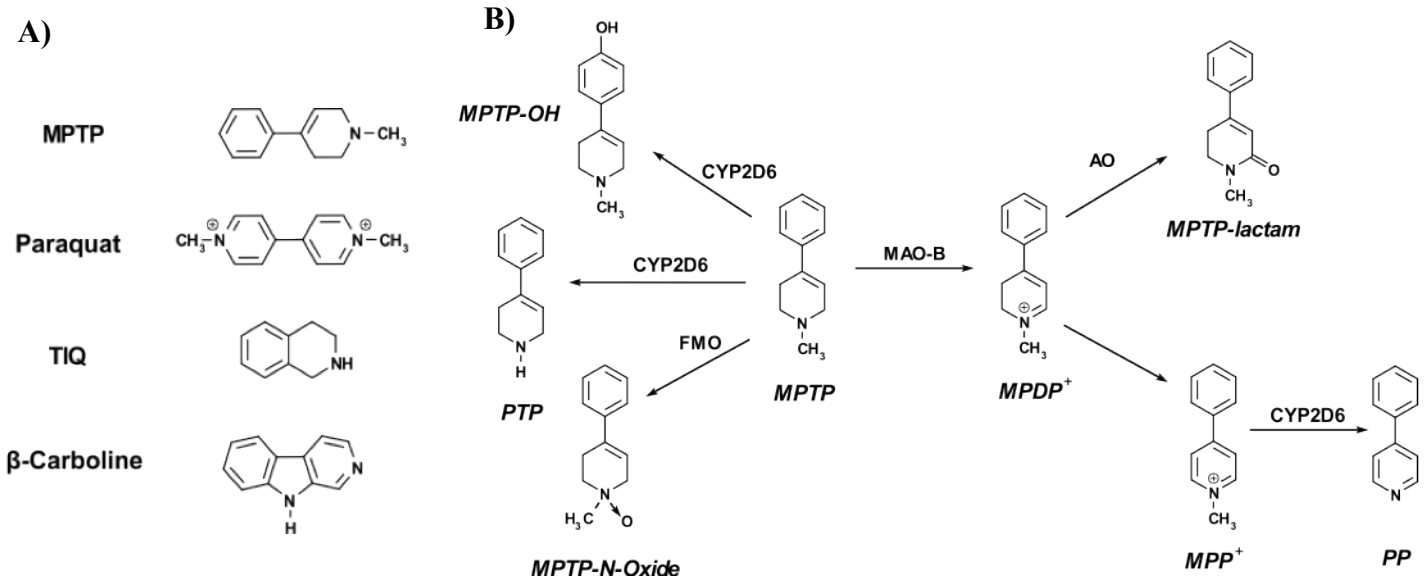


Figure 5: Chemical structures of neurotoxins and MPTP metabolism
A) The structural resemblance between exogenous and endogenous neurotoxins. **B)** Schematic of MPTP and its metabolic pathways in the liver. This figure is adapted and modified from Yoshihara et al., (2000). AO, aldehyde oxidase; FMO, flavin monoamine oxidase.

Endogenous compounds that are similar to MPTP in chemical structure include isoquinolines and β-carbolines (**Figure 5A**). Isoquinolines and β-carbolines are commonly

found in plants and specific foods (e.g., bananas, milk, burnt foods); they are lipophilic and can easily cross the BBB (Nagatsu, 1997). They are markedly higher in the cerebral spinal fluid of PD cases than in controls, suggesting they may be involved in the pathogenesis of PD (Kuhn *et al.*, 1996; Maruyama *et al.*, 1996). In particular, isoquinolines such as TIQ and its derivatives (salsolinol and 1-benzyl-TIQ) are much higher in PD cases than in controls (Kotake *et al.*, 1995; Naoi *et al.*, 2004). Furthermore, TIQ can evoke parkinsonian pathology and behavioural symptoms in monkeys and rodents at high doses (50-100 mg/kg) administered over a long duration (up to 102 day) (Nagatsu & Yoshida, 1988; Yoshida *et al.*, 1990; Yoshida *et al.*, 1993; Lorenc-Koci *et al.*, 2006). Notably, isoquinolines are neurotoxic *in vitro* and *in vivo*, but are much weaker neurotoxins than paraquat, rotenone, and MPTP (Kotake *et al.*, 1996; Mellick *et al.*, 1999b; Shavali *et al.*, 2004; Saitoh *et al.*, 2006; Kotake *et al.*, 2007). The co-administration of isoquinolines with a toxin (e.g., 6-OHDA) can exacerbate neurotoxicity (Kwon *et al.*, 2010). The neurotoxic effects elicited by isoquinolines are proposed to be through the inhibition of mitochondrial complex I (Morikawa *et al.*, 1998; Storch *et al.*, 2000; Patsenka & Antkiewicz-Michaluk, 2004).

As with TIQ, β-carbolines are less potent than other PD-neurotoxins (e.g., rotenone, MPTP) (McNaught *et al.*, 1996). Some β-carbolines are substrates of the DA transporter (DAT), have a tendency to accumulate in the mitochondria, and can function as potent inhibitors of mitochondrial complex I (Riederer *et al.*, 2002; Wernicke *et al.*, 2007). Tetrahydro-β-carbolines, methoxy-β-carbolines, and norharman have been shown to induce toxicity both *in vitro* and *in vivo*, and recently norharman was shown to exacerbate the effects of 6-OHDA in rats in much the same way that isoquinolines do (Lorenc-Koci *et al.*, 2006; Pavlovic *et al.*, 2006; Haghdoost-Yazdi *et al.*, 2010).

3.2.B.i. MPTP

MPTP (**Figure 5**) is a highly lipophilic potent pro-neurotoxin that results in the loss of dopaminergic neuronal cells and closely mimics PD neurochemistry and pathology (Przedborski, 2007). MPTP can elicit clinical symptoms that are responsive to *L*-3, 4-dihydroxyphenylalanine (*L*-DOPA) treatment, commonly used to treat PD, further substantiating the use of MPTP to model PD (Langston *et al.*, 1984a; Langston *et al.*, 1984b). MPTP can elicit some motor dysfunction in monkeys, rats, mice, cats, dogs, and guinea pigs (McCallum *et al.*, 2006b; Przedborski, 2007; Meredith *et al.*, 2008). Of these types of animals, monkeys are the most sensitive to MPTP and display similar pathological and motor responses as observed in PD patients (Przedborski, 2007; Capitanio & Emborg, 2008).

MPTP is metabolized to $MPDP^+$ by monoamine oxidase (MAO)-B in glial cells and subsequently converted to MPP^+ by peroxidases or spontaneous deprotonation (**Figure 5B**) (Notter *et al.*, 1988; Chiba *et al.*, 1995). MPP^+ is then transported into DA neurons by the DAT and rapidly accumulates in the mitochondria (Notter *et al.*, 1988; Herkenham *et al.*, 1991; Fukuda, 2001). The primary mechanism of cell death by MPP^+ is through the inhibition of mitochondrial complex I. However MPP^+ can also promote ROS formation, change mitochondrial membrane permeability, displace DA from vesicles, increase DA release, compete for the DAT, activate caspase-3, and inhibit tyrosine hydroxylase and MAO enzymes (Singer *et al.*, 1986; Notter *et al.*, 1988; Fukuda, 2001; Bordia *et al.*, 2006; Zeng *et al.*, 2006). Studies on the effects of MPTP have provided significant insight into potential mechanisms for environmental neurotoxicity. They have also highlighted some key factors (e.g., DAT, MAO) that influence an individuals' sensitivity to neurotoxins, as a result of genetic variation in these protiens influencing their expression levels and/or activity (Cordato & Chan, 2004).

3.2.C Environment and genetics

It is becoming widely accepted that there is interplay between genetics and environment in the etiology of sporadic PD (Vance *et al.*, 2010). For example, α-syn expression increases *in vivo* upon exposure to paraquat, and neurotoxin-induced damage is exacerbated in *SNCA* over-expressing transgenic mice compared to control mice (Gatto *et al.*, 2010; Horowitz & Greenamyre, 2010).

Proteins directly involved in the transport (e.g., DAT) or metabolism (e.g., MAO-B) of neurotoxins may also play a role in increased PD risk (Mellick *et al.*, 1999a; Cordato & Chan, 2004). Some individuals may be more susceptible to environmental and/or endogenous neurotoxins than are other individuals through altered transport or metabolism of these compounds. Enzymes (e.g., CYPs) can display a great degree of genetic variability in protein expression and function, which may contribute to differing risk for PD. Enzymes involved in the activation or inactivation of MPTP include MAO, flavin monooxygenase, aldehyde oxidase, CYP3A4, CYP1A2, and CYP2D6 (**Figure 5B**) (Chiba *et al.*, 1995; Yoshihara *et al.*, 2000; Mushiroda *et al.*, 2001). MAO has been shown to activate neurotoxins such as MPTP (**Figure 5B**) and β-carbolines, while CYP2D6, CYP1A2, and CYP3A4 have been shown to inactivate MPTP and metabolize various pesticides, including chloropyrifos and atrazine (Coleman *et al.*, 1996; Coleman *et al.*, 2000a; Herraiz *et al.*, 2006; Herraiz *et al.*, 2008).

3.2.C.i Monoamine oxidase and neurotoxicity

MAOs are flavin adenosine dinucleotide-containing enzymes located in the outer membrane of the mitochondria (Binda *et al.*, 2004). MAO is expressed in most tissues, but the highest expression is found in the liver and in the brain (Fowler *et al.*, 2000; Lewis *et al.*, 2007). There are two forms of this enzyme, MAO-A and MAO-B, which have differing affinities towards substrates and differing cellular expressions in the brain (Binda *et al.*, 2006).

MAO-B is more abundantly expressed in the human brain, especially in the basal ganglia, than MAO-A and is expressed in serotonergic neurons, glia, and astrocytes (Binda *et al.*, 2006). MAO-A is expressed mostly in catecholaminergic neurons (Saura *et al.*, 1996). In the brain, these enzymes catalyze the oxidative deamination of exogenous amines (e.g., tyramine) and endogenous biogenic amines to control and regulate monoamine transmitter concentrations (e.g., DA) (Abell & Kwan, 2001).

MAO-B genetic variants that result in higher enzymatic activity are associated with increased risk for PD; this is consistent with its ability to activate neurotoxins (e.g., MPTP, β-carbolines) and metabolize DA to form the oxidative stress induced product H_2O_2 (Singer *et al.*, 1986; Coleman *et al.*, 1996; Chinta & Andersen, 2005; Bialecka *et al.*, 2007; Dick *et al.*, 2007; Singh *et al.*, 2008). Conversely, MAO inhibitors (e.g., selegiline) enhance neuroprotection in *in vitro* and *in vivo* models of PD (Langston *et al.*, 1984c; Castagnoli *et al.*, 2003; Binda *et al.*, 2006). Notably, MAO increases with age and age is a leading risk factor for PD (Fowler *et al.*, 2000; Fahn, 2010). This increase in expression may contribute to an increase in the activation of toxins and lead to oxidative damage and cell death.

3.2.C.ii CYP2D6 and neurotoxicity

CYP2D6 can metabolize trace amines and inactivate neurotoxins such as MPTP and β-carbolines (Coleman *et al.*, 1996; Yu *et al.*, 2003c). Coleman et al. (1996) reported a large variability (0–90%) in the extent of MPTP inactivation to PTP by CYP2D6 (**Figure 5B**) in human liver microsomes. This large variation in CYP2D6 inactivation of toxins may lead to differences in neurotoxicity. However, while the liver plays a significant role in toxin clearance, CYP2D6 in the brain may also contribute to localized neurotoxin inactivation. Using microdialysis, Vaglini et al. (2004) demonstrated that a large proportion of MPTP entering into the striatum resulted in the formation of PTP, MPTP *N*-oxide, $MPDP^+$, and MPP^+. The MPTP

metabolite PTP is largely synthesized by CYP2D6 through *N*-demethylation (Coleman *et al.*, 1996; Gilham *et al.*, 1997; Herraiz *et al.*, 2006). The detection of $MPDP^+$, MPP^+, and PTP metabolites *in vitro* in the presence of both cDNA expressed CYP2D6 and MAO suggests PTP formation in the brain could result from localized MPTP detoxification by CYP2D6 (Herraiz *et al.*, 2006).

A study comparing MPTP toxicity in different rat strains found that female Dark Agouti rats (models of CYP2D6 poor metabolizers) showed more pronounced effects of MPTP and a longer reduction of motor activity compared to other female rat strains (Jimenez-Jimenez *et al.*, 1991). Another study found that the over-expression of CYP2D6 in PC12 cells provided protection from MPP^+ and, to a lesser extent, MPTP (Matoh *et al.*, 2003). Together, these studies suggest that low levels of CYP2D6 reduce neuroprotection and high levels increase it.

3.2.C.iii CYP2D6 and Parkinson's disease

The association of CYP2D6 and PD was first reported by Barbeau et al. when they observed that a number of PD cases were PMs for debrisoquine (Barbeau *et al.*, 1985). Since then, over 30 epidemiological studies have been conducted on CYP2D6 and PD risk, investigating variation in both CYP2D6 genotype and phenotype. Some studies indicate that non-functional CYP2D6 can increase the risk for PD (Deng *et al.*, 2004; Duric *et al.*, 2007; Singh *et al.*, 2010). Two meta-analyses reported overall odds ratios of 1.47 (95% CI: 1.18-1.96) and 1.48 (95% CI: 1.10-1.99) for PD in CYP2D6 PMs (McCann *et al.*, 1997; Christensen *et al.*, 1998). When restricting the meta-analysis to *CYP2D6* genetic studies, the odds ratio for PD changed from 1.48 (95% CI: 1.10-1.99) to 1.67 (95% CI: 1.11-2.50) (Christensen *et al.*, 1998). Moreover, when *CYP2D6* PMs had pesticide exposure their risk for PD further increased, suggesting an interaction of the *CYP2D6* gene and environment (Deng *et al.*, 2004; Elbaz *et al.*, 2004). Overall, these studies indicate that individuals with high CYP2D6 (EMs)

are protected against PD compared to those with low CYP2D6 (PMs), especially when exposed to environmental neurotoxins. These findings, coupled with the ideal expression of CYP2D6 in the brain to metabolize endogenous and exogenous neurotoxins (e.g., TIQ, β-carbolines, pesticides), further supports the premise that CYP2D6 may contribute to protection against neurotoxins and PD.

4. Neuroprotection with smoking and nicotine

In contrast to environmental factors that increase the risk for PD (e.g., pesticides) or elicit a parkinsonian syndrome, there are factors that reduce the risk of developing PD. Neuroprotection can be considered a mechanism of reducing cell dysfunction, cell damage, and/or cell death (Linazasoro, 2009). In the context of PD, neuroprotection can be thought of as an intervention that would prevent neuronal damage from occurring, slow neuronal damage that has been initiated, repair injured neurons, and/or protect and strengthen surviving neurons. Some neuroprotective candidates shown to reduce the risk for PD include cigarette smoking, caffeine, non-steroidal anti-inflammatory drugs, and, to some extent, alcohol (Benedetti *et al.*, 2000; Powers *et al.*, 2008). Factors shown to be neuroprotective both in *in vitro* and *in vivo* PD models include nicotine, MAO-B inhibitors, coenzyme Q10, antioxidants, chelators, and melatonin (Singh *et al.*, 2007; Spindler *et al.*, 2009).

4.1 Smoking and Parkinson's disease

Since first reported by Dorn in 1959, cigarette smoking is one factor that has repeatedly been shown to protect individuals from developing PD (Dorn, 1959; Tanner, 2010). Over 40 studies show smoking reduces the relative risk of getting PD by ~50% compared to age- and gender-matched controls (Allam *et al.*, 2004; Alves *et al.*, 2004; Kandinov *et al.*, 2007). A similar trend in neuroprotection by smoking has been observed in individuals known to be exposed to pesticides (Galanaud *et al.*, 2005). However, while smoking reduces the risk for

developing PD, it does not affect disease progression (Alves *et al.*, 2004; Kandinov *et al.*, 2007; 2009).

Cigarette smoking displays a temporal relationship with PD risk; the duration of smoking and the age at quitting both show a dose-dependent decrease in risk. Specifically, the more years an individual has smoked, the more cigarettes smoked per day, and the fewer years (if any) that have passed since quitting all significantly contribute to a dose-dependent lowering of risk for PD (Thacker *et al.*, 2007). The dose-dependent effect is observed in both genders, even though males have a higher PD risk than women (Allam *et al.*, 2007; Ritz *et al.*, 2007). Twin studies also show this inverse relationship between cigarette pack-years and the onset of PD (Tanner *et al.*, 2002). The relationship was more evident in monozygotic twins than in dizygotic twins, and as monozygotic twins are genetically identical, the protection observed with smoking is unlikely a result of genetic factors or environmental confounders. Neuroprotection by tobacco is further supported by evidence of an inverse relationship between smokeless tobacco and PD (Ritz *et al.*, 2007).

The inverse relationship between smoking and PD has been challenged multiple times, especially by the idea of selective mortality in smokers. However, a 30-year prospective cohort involving 8,000 men showed that smoking was less prevalent in those who developed PD, with an adjusted relative risk of 0.4 (95% CI 0.3-0.6) (Morens *et al.*, 1996). The report noted that protection against PD in smokers was observed at every age group both above and below the age of 60 years (the average on-set for PD). Additionally, there was only a small difference in mortality between smokers and non-smokers before the age of 60, which suggests that it is not the large selective mortality in smokers prior to onset of average age of PD that explains the low prevalence of smokers in the PD population. Other reports indicate mortality rates in PD smokers are either similar or reduced compared to PD non-smokers (Alves *et al.*, 2004;

O'Reilly *et al.*, 2005). These results combined with the observation of a dose-dependent relationship of years smoked, number of cigarettes smoked per day (heavy *versus* light), and number of years (if any) that have passed since quitting strongly affirms the idea that smoking is protective against PD (Gorell *et al.*, 1999; Hernan *et al.*, 2001). Overall, the epidemiological evidence strongly suggests that cigarette smoking is playing a neuroprotective role, but the mechanism is still under investigation.

4.1.A Smoking and neuroprotection

The neuroprotection by cigarette smoking observed in humans also extends to *in vivo* studies where mice exposed to cigarette smoke were protected against MPTP-induced dopaminergic neuronal degeneration (Parain *et al.*, 2003). The neuroprotection by smoking is likely mediated by one or more of the numerous chemicals found in cigarette smoke, including nicotine (discussed in **Section 4.2**). While the exact mechanism is unknown, changes caused by smoking within the dopaminergic and cholinergic systems are thought to be involved in its neuroprotection against PD (Quik, 2004).

Recent imaging studies show that cigarette smokers have decreased DAT availability compared to non-smokers especially in the striatum an area that exhibits the most damage during PD (Newberg *et al.*, 2007; Yang *et al.*, 2008). Compared to other brain structures the striatum is one of the highest expressing DAT regions, where the loss of DAT correlates with nigrostriatal neuronal loss in animal models of PD (Shih *et al.*, 2006; Troiano *et al.*, 2009). The DAT is involved in the uptake of DA, the neurotoxin MPP^+, and select derivatives of the endogenous and exogenous neurotoxins isoquinolines and β-carbolines. MPP^+ uptake by the DAT confers its selectivity of dopaminergic neuronal damage, and is thought to be involved in the selective damage by endogenous and/or exogenous neurotoxins (Storch *et al.*, 2004a; Storch *et al.*, 2004b). *In vitro* studies indicate a positive correlation between DAT expression

and neurotoxicity from β-carbolines (Wernicke *et al.*, 2007). Additionally, variants in DAT show an increased risk for PD that is amplified with exposure to pesticides (Ritz *et al.*, 2009). Altogether, the reduction in DAT by cigarette smoke may be one mechanism of neuroprotection elicited by smoking.

A leading theory of how smoking may be neuroprotective is via MAO inhibition. Smokers have peripheral MAO inhibition, and in the brain they have ~20% inhibition of MAO-A and ~50% inhibition of MAO-B compared to non-smokers and former smokers (Berlin *et al.*, 2000; Fowler *et al.*, 2000; Fowler *et al.*, 2003; Fowler *et al.*, 2005). *In vitro* studies confirm that substances in cigarette smoke, including TIQ derivatives and β-carbolines, can inhibit MAO activity (Castagnoli *et al.*, 2003). Of the β-carbolines in cigarette smoke, tetrahydro-β carbolines, harman, and norharman can inhibit MAO and are elevated in the plasma of smokers (Rommelspacher *et al.*, 2002). *In vivo* studies show some MAO inhibitors in cigarette smoke (e.g., 2,3,6-trimethyl-1 and 4-naphthoquinone) protect against MPTP neurotoxicity in mice (Castagnoli *et al.*, 2001; Castagnoli *et al.*, 2003). As MAO enzymes can activate neurotoxins (i.e., MPTP to MPP^+; **Figure 5**) and can contribute to oxidative stress, the inhibition of MAO by cigarette smoke may assist in reducing neurotoxicity.

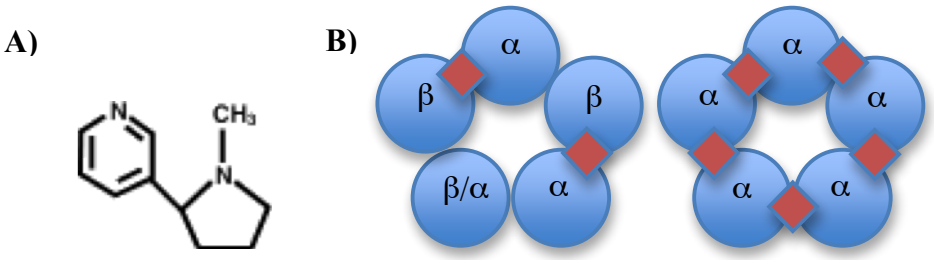
4.1.B Smoking and brain CYPs

Another potential mechanism for the protection mediated by cigarette smoking against PD is through the induction of CYPs in the liver and/or brain that can inactivate neurotoxins, such as pesticides (Miksys & Tyndale, 2006). Smokers have higher CYP2B6 and CYP2E1 levels in the brain compared to non-smokers (Howard *et al.*, 2003; Miksys *et al.*, 2003). CYP2B6 can metabolize a number of pesticides and can detoxify herbicides, such as pyributicarb and trifluralin; therefore, it may inactivate these chemicals locally in the brain (Lang *et al.*, 1997; Tang *et al.*, 2002; Hirose *et al.*, 2005; Kawahigashi *et al.*, 2005). CYP2E1 is

ideally situated in dopaminergic neurons of the SN but is minimally involved in pesticide metabolism (Riedl *et al.*, 1996; Watts *et al.*, 1998; Hodgson, 2001). However, selective inhibition of CYP2E1 has been shown to increase MPTP-induced neurotoxicity *in vivo,* suggesting that CYP2E1 may participate in neuroprotection against PD-causing toxins (Vaglini *et al.*, 2004; Viaggi *et al.*, 2006). Altogether, higher levels of brain CYP2B6, and possibly CYP2E1, in smokers may help to protect against pesticide or neurotoxin elicited damage. Nicotine is believed to be one agent in cigarette smoke that can induce these CYPs in the brain (Joshi & Tyndale, 2006b; Lee *et al.*, 2006a).

4.2 Nicotine

Nicotine (**Figure 6A**) is the most extensively studied neuroprotective agent in cigarette smoke (Belluardo *et al.*, 2000; Quik *et al.*, 2007a). Nicotine can readily cross the BBB and once in the brain it can enhance the release of neurotransmitters (e.g., DA) and can activate diverse pathways that lead to increased neurotropic factors and changes in apoptotic signalling (Quik *et al.*, 2007a; Benowitz, 2008). Nicotine has been shown repeatedly to neuroprotect in various toxin-induced *in vitro* and *in vivo* models of PD (Visanji *et al.*, 2006; Khwaja *et al.*, 2007; Quik *et al.*, 2007c; Riveles *et al.*, 2008). Overall, these effects of nicotine are believed to occur through both receptor-independent and receptor-mediated pathways.



Nicotine Nicotinic acetylcholine receptor

Figure 6: Chemical structure of nicotine and nAChR structure

A) Chemical structure of the alkaloid nicotine, 3-(1-methyl-pyrrolidin-2-yl)-pyridine. **B)** The pentameric structures of the heteromeric (left) and homomeric (right) nAChRs with diamonds depicting the binding sites.

4.2.A Nicotine receptors

Select ligands that interact with different nicotinic acetylcholine receptor (nAChR) subtypes (**Figure 6B**) suggest some neuroprotective effects elicited by nicotine occur downstream of these receptors (Belluardo *et al.*, 2000; Quik *et al.*, 2007a). NAChRs are ligand-gated ion channels expressed on cholinergic as well as non-cholinergic neurons including dopaminergic, serotonergic, glutamatergic, and gamma-aminobutyric acid neurons (Quik *et al.*, 2007a; Lendvai & Vizi, 2008; Yakel, 2010). NAChRs are hetero- or homo-pentameric structures (**Figure 6B**) that occur in various combinations of 8 subunits comprised of α2-α10 and β2-β4 (Gotti & Clementi, 2004). Agonists and antagonists selective for heteromeric α4β2 or homomeric α7 nAChRs demonstrate that the activation of these two receptors can protect from β-amyloid- and rotenone-induced toxicity in cell culture (Kihara *et al.*, 1998; Belluardo *et al.*, 2000; Marrero & Bencherif, 2009; Takeuchi *et al.*, 2009). Additionally, α4β2 knockout mice show decreased neuroprotection by nicotine, further indicating the importance of nAChRs in the neuroprotective properties of nicotine (Ryan *et al.*, 2001). Due to a lack of specific compounds for other nAChR subtypes (other than α4β2 and α7), the individual

involvement of nAChRs in neuroprotection currently remains unknown (Belluardo *et al.*, 2000; Quik & Kulak, 2002; Quik *et al.*, 2007a).

Of particular relevance to PD is the nAChR subtype containing the α6 subunit. Alpha-6 has a very restricted localization and is most abundant in the midbrain dopaminergic systems, including mesocorticolimbic and nigrostriatal pathways (Gotti *et al.*, 1997; Quik *et al.*, 2000; Dome *et al.*, 2010). In the primate striatum, a region that exhibits the greatest damage in PD, the highest expressing receptor subtypes are α6*, α3β2*, and α4β2* (* represents other subunits) (Gotti & Clementi, 2004; Quik *et al.*, 2007a).

Selective lesioning of the dopaminergic nigrostriatal pathway with MPTP in monkeys, and with paraquat in mice, results in a significant decrease of α6β2* followed by α4β2* nAChRs that parallels the loss of DAT (Quik *et al.*, 2001; Bordia *et al.*, 2006; Khwaja *et al.*, 2007). Similarly, in PD cases the α6β2* subtype is notably decreased with a small decrease in α4β2* compared to age-matched controls (Gotti & Clementi, 2004; Quik, 2004; Bordia *et al.*, 2007; Quik *et al.*, 2007a). Given the selective expression of α6β2* subtype on vulnerable neuronal populations this nAChR subtype could potentially be a specific drug target for PD neuroprotection.

In monkeys with MPTP-induced nigrostriatal damage, nicotine treatment can prevent the loss of nAChR α4β2* and α3/α6β2* subtypes (McCallum *et al.*, 2006b). Further, nicotine treatment can normalize aberrant dopaminergic activity resulting from nigrostriatal damage and restore synaptic plasticity (McCallum *et al.*, 2006b; Quik *et al.*, 2006a; Quik *et al.*, 2006b). The preservation of nAChRs by nicotine is thought to further contribute to nicotine's neuroprotection, especially neuroprotection elicited by the activation of different downstream pathways and processes (Wonnacott *et al.*, 2005; Bordia *et al.*, 2006).

4.2.B Nicotine and neuroprotection

Nicotine-mediated neuroprotection has been observed in glutamate-, β-amyloid-, and neurotoxin-induced (MPP^+, paraquat, rotenone) toxicity in both primary neuronal cells and other cell models (Quik & Kulak, 2002; Zhang *et al.*, 2006). Chronic nicotine treatment can protect against nigrostriatal damage in MPTP-lesioned monkeys. Specifically, nicotine substantially preserves and/or restores dopaminergic markers that decline with MPTP treatment, including tyrosine hydroxylase, DAT, vesicular monoamine transporter, and DA levels (McCallum *et al.*, 2006b; Quik *et al.*, 2006a; Quik *et al.*, 2006b).

Nicotine has been shown to reduce nitric oxide synthase expression and NO formation *in vitro* (Gatto *et al.*, 2000). PD patients have notably higher levels of NO (a contributor to ROS formation) in their cerebral spinal fluid, and, post-mortem, PD brain tissues display higher levels of neuronal and inducible nitric oxide synthase compared to controls (Mazzio *et al.*, 2005; Aquilano *et al.*, 2008). One mechanism of nicotine neuroprotection may be through the reduction of NO formation and resultant oxidative damage.

Nicotine can also increase ubiquitin-proteasome activity through nAChR activation, which could ultimately reduce the accumulation of misfolded/damaged proteins that contribute to neuronal toxicity (Kane *et al.*, 2004; Chapman, 2009). Importantly, nicotine can increase trophic factors (e.g., fibroblast growth factor-2, brain-derived neurotrophic factor), reduce inflammatory cytokines (e.g., decreases in tumour necrosis factor and interleukin-1), and inhibit apoptotic signalling (e.g., Bcl-2, caspase) (Belluardo *et al.*, 2000; Mai *et al.*, 2003; Quik, 2004). Together, the activation of nAChR and the above-mentioned pathways likely lead to a reduction in toxin-induced apoptosis and promotes neuronal preservation, survival, and/or regeneration.

In addition to protective effects of nicotine through the activation of nAChR, nicotine can elicit protection via non-receptor-mediated mechanisms. *In vitro*, nicotine is a potent antioxidant; it is a scavenger for free radicals and has chelating properties (Ferger *et al.*, 1998; Xie *et al.*, 2005). Nicotine can directly interact with β-amyloid protein and inhibit β-amyloid aggregate formation (Zamani & Allen, 2001; Zhang *et al.*, 2006). Further, nicotine can directly interact with mitochondrial proteins and alter mitochondrial function. For example, nicotine can bind to complex I, inhibit cytochrome *c* release induced by MPP^+, reduce mitochondrial swelling, and prevent ROS generation (Cormier *et al.*, 2001; Xie *et al.*, 2005).

4.2.C Nicotine and Brain CYPs

Similar to the effects seen with cigarette smoking on brain CYP expression, chronic treatment with nicotine can regulate brain CYP2B6, CYP2D6, and CYP2E1. The induction by nicotine occurs in a CYP-, organ-, brain region- and cell-specific manner. As with smoking, nicotine can induce CYP2B6 in the frontal cortex of monkeys (Lee *et al.*, 2006a). Notably, while CYP2E1 is abundantly expressed in the SN of monkeys, it is not induced by nicotine in this region but is induced in the frontal cortex and cerebellum (Joshi & Tyndale, 2006b). In rats, nicotine can induce CYP2D in the striatum and cerebellum, as well as in select cells of the frontal cortex (pyramidal neurons) and hippocampus (granular cells) (Yue *et al.*, 2008). These induced levels of brain CYPs, especially CYP2D6, may contribute to the localized inactivation of toxins, and may be one additional mechanism of neuroprotection that has been observed with smoking as well as with nicotine.

Study rationales and statement of research hypotheses

Chapter 1: Cytochrome P450 2D6 enzyme neuroprotects against 1-methyl-4-phenylpyridinium toxicity in SH-SY5Y neuronal cells

The ability of CYP2D6 to inactivate a number of endogenous and exogenous neurotoxins, along with its ideal localization in select brain regions (i.e., substantia nigra and striatum) and cell types (i.e., dopamine neurons and glial cells) affected in PD, make CYP2D6 a likely candidate to study in a PD model. If the degree of toxicity were influenced by the balance between activation (MPTP to MPP^+ by monoamine oxidase-B) and inactivation (MPTP to PTP and MPP^+ to PP by CYP2D6) of neurotoxins, then low levels of brain CYP2D6 would decrease neurotoxin inactivation and reduce neuroprotection. Evidence supporting this rationale is found in epidemiological studies that show CYP2D6 PMs are at higher risk for PD compared to CYP2D6 EMs (Singh *et al.*, 2010). As there is little evidence supporting the direct involvement of CYP2D6 in neuroprotection against PD causing agents, the first goal was to establish this involvement by using endogenously expressed CYP2D6 in a cultured neuronal cell model of PD. **I hypothesize that inhibiting CYP2D6 function in SH-SY5Y human neuroblastoma cells will increase MPP^+-induced neurotoxicity.**

Chapter 2: Induction of the drug metabolizing enzyme CYP2D in monkey brain by chronic nicotine treatment

In **Chapter 1**, we show that functionally inhibiting CYP2D6 in SH-SHY5Y human neuroblastoma cells results in increased MPP^+-mediated neurotoxicity. As over-expression of CYP2D6 can protect against MPP^+ induced cytotoxicity in PC12 cells, then induced or high levels of brain CYP2D6 are likely to increase neuroprotection (Matoh *et al.*, 2003). These results, in addition to the known expression of CYP2D6 in areas affected in PD (e.g., the substantia nigra and basal ganglia), suggest that functional brain CYP2D6 may be playing a role in localized neurotoxin inactivation and thereby protecting against PD. Previous studies have shown CYPs can be up- or down-regulated by xenobiotics in an organ- and brain region-specific manner (Miksys & Tyndale, 2004). The neuroprotective chemical nicotine, for example, can induce CYP2D in the rat brain (Yue *et al.*, 2008). Because smokers are protected against developing PD, nicotine is neuroprotective in models of PD, and high CYP2D6 levels may be neuroprotective, we determined if cigarette smokers have higher brain CYP2D6 and if nicotine could induce brain CYP2D6 in primates. **We hypothesize that 1) smokers will have higher brain CYP2D6 compared to non-smokers, and 2) chronic treatment with nicotine will induce brain CYP2D in monkeys.**

Chapter 3: The neuroprotective enzyme CYP2D6 increases in the brain with age and is lower in Parkinson's disease patients.

The exact etiology of PD is not yet clearly understood, however both genetic and environmental influences contribute. CYP2D6 is one promising neuroprotective candidate because it can inactivate neurotoxins (e.g., pesticides) and is genetically highly polymorphic. Some genetic studies have shown an increased risk for PD in *CYP2D6* PMs (McCann *et al.*, 1997; Deng *et al.*, 2004; Elbaz *et al.*, 2004). The susceptibility for PD in *CYP2D6* PMs is further increased when they are exposed to pesticides, supporting a gene and environmental interaction for *CYP2D6* and PD risk (Deng *et al.*, 2004).

In **Chapter 1**, we demonstrate that lowering levels of functional CYP2D6 reduce neuroprotection from the damaging effects of MPP^+. In **Chapter 2**, we show that brain CYP2D6 is higher in cigarette smokers compared to non-smokers and induced by nicotine in primates. As both cigarette smoking and nicotine are neuroprotective, the elevated levels of CYP2D6 may be one mechanism of their neuroprotection against PD. These studies collectively suggest that the levels of brain CYP2D6 may influence CNS inactivation of neurotoxins thereby altering the effects of neurotoxin-induced damage on PD risk.

CYP2D6 displays large interindividual variability, and this variation may render some individuals more or less prone to the effects of endogenous and/or exogenous neurotoxins. Evidence from **Chapter 1** and previous studies looking at CYP2D6 genetics and PD risk suggest that functionally low CYP2D6 levels reduce neuroprotection, and therefore determined if PD cases also express lower brain CYP2D6 protein. **We hypothesize that PD cases will have lower brain CYP2D6 protein compared to age-matched controls.** Because PD is an age-related neurological disease, we also assessed if the levels of brain CYP2D6 protein change with age. As hepatic CYP2D6 increases only from birth to one year of age, **we**

hypothesize that brain CYP2D6 protein will increase from birth to one year without further change through adulthood.

Section 2: Thesis Chapters

Chapter 1: Cytochrome P450 2D6 enzyme neuroprotects against 1-methyl-4-phenylpyridinium toxicity in SH-SY5Y neuronal cells

Amandeep Mann and Rachel F. Tyndale

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Dr. Rachel F. Tyndale and Amandeep Mann designed the neurotoxicity study. Amandeep Mann 1) conducted all the experiments, 2) preformed the data analysis, and 3) wrote the manuscript. Dr. Rachel F. Tyndale helped with writing and editing the manuscript.

Abstract

Cytochrome P450 (CYP) 2D6 is an enzyme that is expressed in liver and brain. It can inactivate neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, 1,2,3,4-tetrahydroisoquinoline and β-carbolines. Genetically slow CYP2D6 metabolizers are at higher risk for developing Parkinson's disease, a risk that increases with exposure to pesticides. The goal of this study was to investigate the neuroprotective role of CYP2D6 in an *in vitro* neurotoxicity model. SH-SY5Y human neuroblastoma cells express CYP2D6 as determined by western blotting, immunocytochemistry and enzymatic activity. CYP2D6 metabolized 3-[2-(*N*, *N*-diethyl-*N*-methylammonium) ethyl]-7-methoxy-4-methylcoumarin and the CYP2D6-specific inhibitor quinidine (1 μM) blocked 96 ± 1% of this metabolism, indicating that CYP2D6 is functional in this cell line. Treatment of cells with CYP2D6 inhibitors (quinidine, propranolol, metoprolol or timolol) at varying concentrations significantly increased the neurotoxicity caused by 1-methyl-4-phenylpyridinium (MPP^+) at 10 and 25 μm by between 9 ± 1 and 22 ± 5% ($P < 0.01$). We found that CYP3A is also expressed in SH-SY5Y cells and inhibiting CYP3A with ketoconazole significantly increased the cell death caused by 10 and 25 μm of MPP^+ by between 8 ± 1 and 30 ± 3% ($P < 0.001$). Inhibiting both CYP2D6 and CYP3A showed an additive effect on MPP^+ neurotoxicity. These data further support a possible role for CYP2D6 in neuroprotection from Parkinson's disease-causing neurotoxins, especially in the human brain where expression of CYP2D6 is high in some regions (i.e. substantia nigra).

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder in which both genetic (i.e., Parkin and Leucine-rich repeat kinase 2) and environmental (i.e., pesticides) risk factors have been implicated in the etiology (Di Monte, 2003; Olanow, 2007). One genetic factor associated with a predisposition to injury from exogenous toxins is the drug- and toxin-metabolizing enzyme cytochrome P450 (CYP) 2D6 (McCann *et al.*, 1997). Genetic association studies show that poor metabolizers of CYP2D6, those lacking a functional enzyme, have an increased risk for PD (McCann *et al.*, 1997). This risk is further elevated when these individuals are exposed to pesticides (Deng *et al.*, 2004; Elbaz *et al.*, 2004). This suggests that having inactive CYP2D6 makes individuals more susceptible to the effects of environmental neurotoxins, such as pesticides, which is probably due to their inability to inactivate these neurotoxins.

Cytochrome P450 2D6 is a drug- and toxin-metabolizing enzyme that can inactivate a number of neurotoxins including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Modi *et al.*, 1997), tetrahydroisoquinolines (Suzuki *et al.*, 1992), harmaline, harmine (Yu *et al.*, 2003d) and *N*-methyl-β-carbolines (Herraiz *et al.*, 2006). CYP2D6 is found in the liver and in peripheral tissues including the brain (Miksys *et al.*, 2002; Mann *et al.*, 2008), where it has been shown to be enzymatically functional (Strobel *et al.*, 1995; Tyndale *et al.*, 1999; Voirol *et al.*, 2000; Bromek *et al.*, 2010). *In-situ* inactivation of MPTP to PTP was shown in rat striatum, a reaction largely mediated by CYP2D by *N*-demethylation (Coleman *et al.*, 1996; Vaglini *et al.*, 2004; Herraiz *et al.*, 2006). The expression of CYP2D6 is brain region and cell type specific and also exhibits high interindividual variability (Miksys *et al.*, 2002; Mann *et al.*, 2008). In primates, CYP2D protein levels are highest in the basal ganglia, especially the substantia nigra (Miksys *et al.*, 2002; Mann *et al.*, 2008), consistent with data in rat brain (Bromek *et al.*, 2010). In fact, in the substantia nigra, CYP2D co-localizes with tyrosine

hydroxylase, a marker for dopaminergic neurons (Watts *et al.*, 1998). CYP2D6 is also found in glial cells (Miksys *et al.*, 2000b) that can also express the neurotoxin-activating enzyme monoamine oxidase B (Nicotra & Parvez, 2000; Herraiz *et al.*, 2007). Thus, CYP2D6 is ideally situated in cells and brain regions to locally detoxify PD-causing agents.

In a rodent model, a study comparing the toxicity of MPTP in different rat strains found that female Dark Agouti rats (models of poor metabolizers of CYP2D) showed more pronounced effects of MPTP and a longer reduction of motor activity compared with other strains (Jimenez-Jimenez *et al.*, 1991). Consistent with this, over-expression of CYP2D6 in PC12 cells was shown to protect against 1-methyl-4-phenylpyridinium (MPP^+) cytotoxicity (Matoh *et al.*, 2003). Together, this evidence suggests that the expression of functional CYP2D6 may provide a defense against endogenous and/or exogenous neurotoxins. Given the lack of direct evidence for the involvement of CYP2D6 in neuroprotection, we proposed to test the importance of endogenous CYP2D6 in protecting against MPTP / MPP^+ neurotoxicity in SH-SY5Y cells. The goals of the current study were to (i) determine whether human neuroblastoma SH-SY5Y cells express functional CYP2D6 and (ii) investigate the direct contribution of endogenous CYP2D6 to protection against MPP^+-induced neurotoxicity in this neuronal cell line using CYP2D6 inhibitors.

Materials and methods

Materials

The 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 CYP1A2, CYP2D6, CYP3A4, 3-[2-(*N*,*N*-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride and the 3-[2-(*N*,*N*-diethyl-*N*-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC) high-throughput inhibitor screening kit were purchased from BD Biosciences (Mississauga, ON, Canada). Polyclonal antibody (PAb) raised in rabbit against amino acids 254–273 of CYP2D6 was a gift from A. Cribb and Merck & Co. (Whitehouse Station, NJ, USA) (Cribb *et al.*, 1995; Miksys *et al.*, 2002). CYP1A2 PAb raised in rabbit was purchased from Affinity Bio-Reagents (Golden, CO, USA). CYP3A4 monoclonal antibody was purchased from Diachii Pure Chemicals Co., Ltd (Tokyo, Japan). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies raised in goat were purchased from Chemicon International, Inc. (Temecula, CA, USA). Avidin–biotin complex with peroxidase kit and 3,3'-diaminobenzidine 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). T-175-cm^2 vented cap cell culture flasks were from Sarstedt (Newton, USA). SH-SY5Y cells and the 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit were from American Type Culture Collection (Manassas, VA, USA). Roswell Park Memorial Institute + L-glutamine was purchased from Invitrogen (Burlington, ON, Canada). MPTP, MPP^+, quinidine, propranolol, metoprolol and timolol were purchased from Sigma (Mississauga, ON, Canada).

Cell culture

The SH-SY5Y cells were cultured in 175-cm^2 flasks in Roswell Park Memorial Institute + L-glutamine with 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin and 50 mg/mL streptomyocin in a humidified, 5% CO_2, 37°C incubator. The medium was changed every second day and cells were subcultured at 80% confluency every 4–5 days using 0.1% trypsin. Passages 2–15 were used for all experiments. For immunocytochemistry, cells were cultured at 2 x10^4 cells/well onto poly-D-lysine-coated glass coverslips in six well plates. For the MTT assay, cells were cultured at 1x 10^4 cells/well in 96-well plates. SH-SY5Y cells cultured in 175-cm^2 flasks were harvested and lysed in ice-cold Tris (100 mM, pH 7.4), EDTA (0.1 mM) and dithiothreitol (0.1 mM) buffer and sonicated on ice twice for 10 s. The samples were then either used for western blotting or further purified for activity assays. For activity, cell homogenates were centrifuged at 3000 *g* for 10 min at 4°C. The pellet was resuspended and centrifuged again at 3000 *g* for 10 min at 4 °C. The combined supernatants were centrifuged at 110 000 *g* for 90 min at 4 °C and the pellet resuspended in 100 mM Tris, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1.15% w / v KCl and 20% v / v glycerol. The protein content of the membranes was assayed with the Bradford technique using a Bio-Rad Protein Assay kit. Membranes were either used directly for activity or aliquoted and stored at -80 °C.

Western blotting

The SH-SY5Y whole-cell lysates and cDNA-expressed CYP2D6, CYP1A2 and CYP3A4 were serially diluted to generate standard curves. These standard curves were used to determine the linear detection range and relative amount of each CYP (pmol/μg of whole cell lysate protein) expressed in SH-SY5Y cells. Samples were separated by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis with 8% separating and 4.5% stacking gels. The proteins

were transferred overnight onto nitrocellulose membranes and blocked with 2% w / v skim milk powder in Tris-Buffered Saline Tween-20 (0.1% bovine serum albumin, 0.1% Triton X-100, 50 mM Tris, 150 mM NaCl, pH 7.4). The CYP2D6 PAb used was specific for CYP2D6, based on its lack of cross-reactivity with other human CYPs (Cribb *et al.*, 1995). The blot was probed with CYP2D6 PAb diluted 1 : 3000, CYP3A4 PAb diluted 1 : 4000 or CYP1A2 monoclonal antibody diluted 1 : 10 000 in TBS-T, followed by peroxidase conjugated anti-rabbit or anti-mouse secondary antibody diluted 1 : 3000 or 1 : 17 000, respectively, in TBS-T. Protein was then detected using chemiluminescence. As three different antibodies were used, exposure times varied from 45 s to 5 min for optimal band detection on autoradiographic film. MCID Elite imaging software (Interfocus Imaging Ltd, Linton, UK) was used to analyze the films.

Immunocytochemistry

Cells were grown on poly-D-lysine-coated glass coverslips, blocked for 1 h in 1% w / v skimmed milk, 1% w / v bovine serum albumin, 2% v / v normal horse serum and 0.01% v / v Triton X-100 in phosphate-buffered saline, and then incubated for 48 h at 4 ^{o}C in CYP2D6 PAb at 1 : 500 in phosphate-buffered saline with 0.1% bovine serum albumin

and 2% normal horse serum. The antigen–antibody complex was visualized using biotinylated goat anti-rabbit immunoglobulin (diluted 1 : 500 in phosphate-buffered saline) followed by the avidin–biotin complex technique and reaction with 3,3'-diaminobenzidine and hydrogen peroxide. Negative control sections were incubated in the same manner but without primary antibody.

Conditions for in-vitro metabolism of 3-[2-(N,N-diethyl-Nmethylammonium) ethyl]-7-methoxy-4-methylcoumarin by CYP2D6

The CYP2D6 activity in SH-SY5Y cell membranes was determined by using the specific substrate AMMC (Yamamoto *et al.*, 2003) highthroughput assay screening kit in 96-well microplates. AMMC activity assays were performed as instructed by the kit manufacturer (Gentest, BD Biosciences). In brief, 100 μL of buffer containing a NADPH regenerating system (with final concentrations of 0.013 mM $NADP^+$, 0.6 mM MgCl2, 0.6 mM glucose-6-phosphate and 0.3 U / mL glucose-6-phosphate dehydrogenase) was added to each well. To assess if the metabolism of AMMC was specifically mediated by CYP2D6, a selective CYP2D6 inhibitor, quinidine (0.1–10 μm), was added. The plate was warmed to 37°C for 15 min, followed by the addition of 100 μL of 0.5 M potassium phosphate (pH 7.4) buffer containing AMMC (1.5–5 μm). The reaction was initiated by the addition of prewarmed cDNA-expressed CYP2D6 (1–8 pmol) or SH-SY5Y membranes (50 μg). The plate was then incubated at 37°C for 30 min and the reaction stopped using 75 μL of 0.5 mM Tris base. The fluorescence signal was measured using a SpectroMax Gemini EM (Molecular Devices) fluorescence plate reader at excitation and emission wavelengths of 390 and 450 nm, respectively. 3-[2-(*N,N*-diethylamino) ethyl]-7-hydroxy-4 methylcoumarin hydrochloride (0.03–1000 nM) in buffer was used as the standard for metabolite quantification as recommended for the kit. We confirmed that the fluorescence from quinidine did not interfere with the assay by adding quinidine (0.01–10 μM) to wells with buffer alone. To determine background fluorescence, control wells had enzyme, buffer (with or without inhibitor) and AMMC added after the addition of the stop solution. Specific fluorescence due to AMMC metabolism was determined by subtraction of the fluorescence from these background control wells from the sample fluorescence. In experiments measuring AMMC metabolism by SH-

SY5Y cells, positive control wells contained cDNA-expressed CYP2D6 (2 pmol) plus or minus quinidine (1 μM).

Drug treatments and 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cells were cultured in 96-well plates for 1 day and then pre-treated at 37°C for 30 min with the CYP2D6 inhibitors quinidine (0.01–10 μM), metoprolol (1–100 μM), propranolol (0.1–30 μM), timolol (1–300 μM) or the CYP3A inhibitor ketoconazole (0.0001–10 μM). The cells were treated with MPTP (0.1–3 μM) or MPP^+ (0.01–1 μM) in the continued presence of inhibitors for 48 h and then assessed for cell viability. Cell viability was determined by a mitochondrial enzyme dependent reaction of MTT assay as described by the manufacturer. In brief, 10 μL of MTT was added directly to the culture media with the SH-SY5Y cells. Following a 2-h incubation at 37°C, 100 μL of solubilizing agent was added to dissolve the purple formazan crystals formed by metabolism of the yellow MTT tetrazolium salt. The plate was then incubated for 1 h, or overnight, and the absorbance was measured using the Multiskan Ex (Thermo Electron Co.) plate reader at a wavelength of 570 nm. Cell death following MPP^+ treatment was shown as a percentage of untreated cells. To determine the effect of the inhibitors alone on cell death, cells were treated with each inhibitor at each dose tested in the absence of MPP^+.

Statistical analysis

Data were analyzed using the Self-Propelled Semi-Submersible analytical program (SPSS, Chicago, IL, USA). The comparison of the CYP2D6 metabolism of AMMC in the presence and absence of inhibitor was evaluated using a directional Student's *t*-test. The effect of CYP inhibition on MPTP / MPP^+ neurotoxicity was compared with MPTP / MPP^+-treated cells

without inhibitor, using one-way ANOVA followed by a least significant difference *post-hoc* test. To determine an additive effect of inhibiting both CYP2D6 and CYP3A, a two-way ANOVA was used to test for an interaction between ketoconazole and quinidine on MPP^+ neurotoxicity. This was followed by a one-way ANOVA and least significant difference *post-hoc* test to compare the effects of drug treatments with one another.

Results

SH-SY5Y cells express CYP2D6 protein

Immunocytochemistry indicated that CYP2D6 is expressed throughout the cell including neuronal projections (**Figure 7A**), consistent with *in-situ* neuronal expression (Miksys *et al.*, 2002; Mann *et al.*, 2008). Some partial staining of the nucleus was also seen in negative control cells incubated without primary antibody (**Figure 7A,** bottom left). To quantitatively assess CYP2D6 expression by western blotting, a dilution curve was generated using SH-SY5Y whole-cell lysates (**Figure 7B**). CYP2D6 protein detection (**Figure 7C**) was linear up to 80 μg of SH-SY5Y whole-cell lysate protein and, using a standard curve of cDNA-expressed CYP2D6, it was determined SH-SY5Y cells express 0.29 ± 0.03 pmol of CYP2D6 / μg of whole-cell lysate protein.

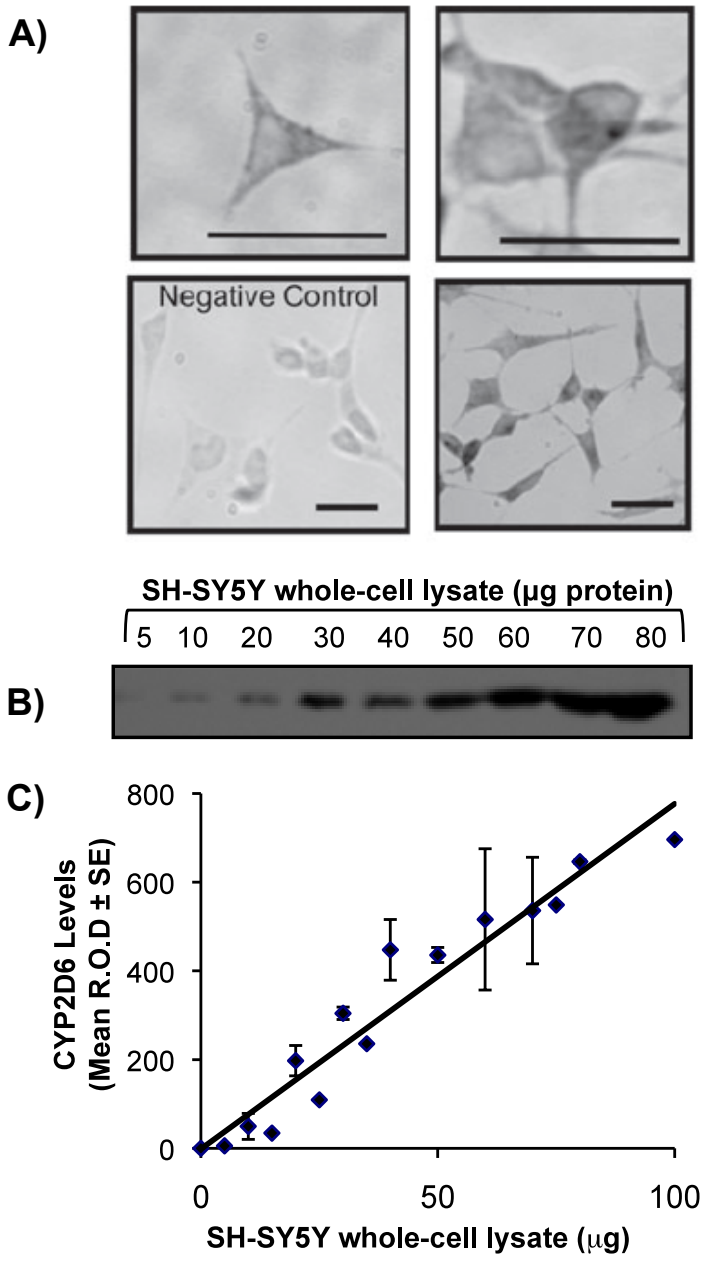

Figure 7: SH-SY5Y cells express CYP2D6 protein
(**A**) CYP2D6 immunohistochemical labeling throughout SH-SY5Y cells. Negative control (bottom left) shows partial staining of the nucleus in the absence of primary antibody. (**B**) A representative immunoblot of CYP2D6 detection in increasing amounts of whole-cell lysate protein. (**C**) A standard curve of CYP2D6 expression [relative optical density (R.O.D.)] in SH-SY5Y cells showing linear detection. Mean band density ± SEM from four western blots. Bar = 100 µm.

CYP2D6 in SH-SY5Y cells is enzymatically active

The metabolism of AMMC to its fluorescent product 3-[2-(*N,N*-diethyl- *N*-methyl-ammonium)ethyl]-7-hydroxy-4-methylcoumarin was dependent on the amount of cDNA-expressed CYP2D6 protein and was inhibited by quinidine (1 μM) (**Figure 8A**). Quinidine dose-dependently inhibited AMMC metabolism by cDNA-expressed CYP2D6 (**Figure 8B**). In agreement with the literature, quinidine at its Ki (0.01 μM) (Bertelsen *et al.*, 2003) inhibited ~50% of AMMC metabolism with complete inhibition at 1 μM or higher. SH-SY5Y cell membranes dose-dependently metabolized AMMC (**Figure 8C**), which was completely inhibited by quinidine (1 μM) ($P < 0.001$). We confirmed that fluorescence from quinidine did not interfere with the assay by adding quinidine (0.01–10 μM) to wells with buffer alone. These results demonstrate a functional CYP2D6 enzyme in SH-SY5Y cells, which can be inhibited by the CYP2D6-specific inhibitor quinidine.

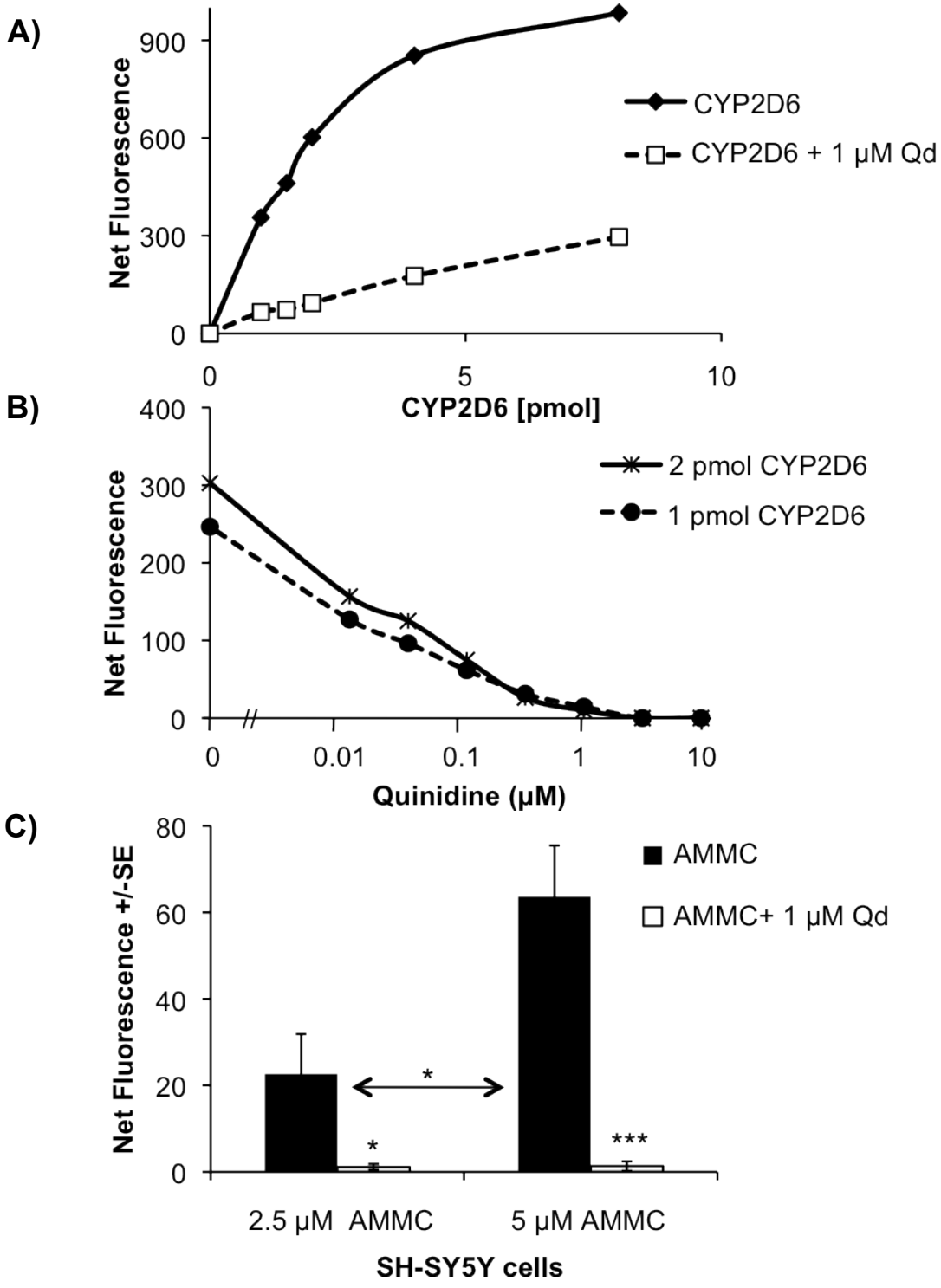

Figure 8: AMMC metabolism by cDNA-expressed CYP2D6 and SH-SY5Y cells
(**A**) Metabolism of AMMC (1.5 µM) is cDNA-expressed CYP2D6 protein dependent and is inhibited by 1 µM of quinidine (Qd). (**B**) Quinidine dose-dependently inhibited cDNA-expressed CYP2D6 metabolism of AMMC (1.5 µM). (**C**) Metabolism of 2.5 and 5 µM AMMC by SH-SY5Y cell membranes (50 µg) in the absence and presence of quinidine (1 µM). Mean ± SEM of two experiments (N = 3 wells / experiment). Results are shown as sample fluorescence with background fluorescence subtracted. Student's *t*-test, **P* < 0.01, ****P* < 0.001.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium neurotoxicity

Both MPTP and MPP^+ induced significant cell death (ANOVA $P < 0.001$) in SH-SY5Y cells (**Figure 9A**). MPP^+ showed a dose dependent effect on cell death. At the highest dose tested, MPTP (3 mM) showed 18 ± 1% cell death compared with 71 ± 3% by MPP^+ (1 mM). MPP^+ caused 18% cell death at ~0.015 μM, suggesting that MPP^+ is 200 times more potent than MPTP in this cell line. Quinidine (0.1 μM) significantly increased cell death by MPP^+ at all doses and at 1 mM of MPTP (**Figure 9B**). For subsequent experiments, 48 h exposure to MPP^+ at low doses was used instead of MPTP because (i) MPP^+ was more potent at causing neurotoxicity and (ii) MPP^+ was significantly affected by 0.1 μM of quinidine (which specifically targets CYP2D6).

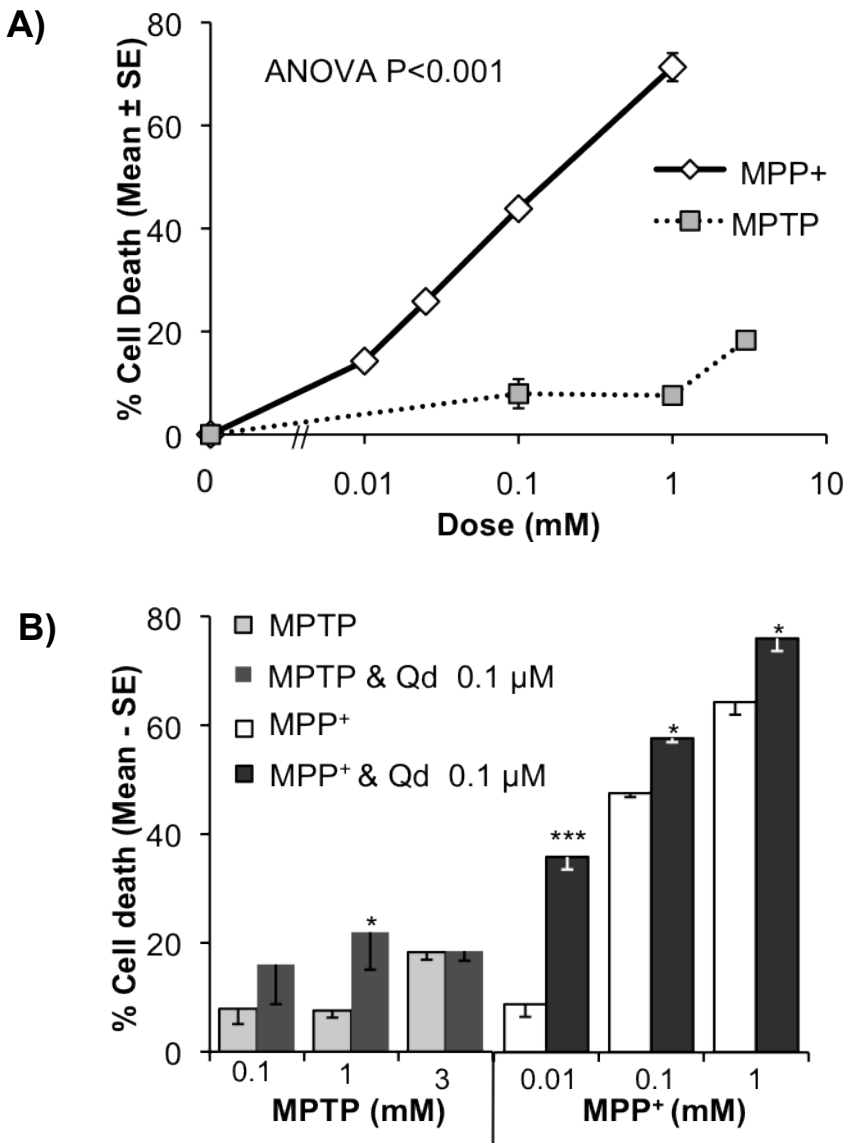

Figure 9: MPTP and MPP^+ neurotoxicity in SH-SY5Y cells.
(**A**) Dose response of 48-h exposure to MPTP and MPP^+ on cell death. (**B**) The effects of CYP2D6 inhibition by quinidine (Qd) (0.1 µM) on MPTP and MPP^+ neurotoxicity. Mean of three wells per toxicity group ± SEM. Results are shown as percent cell death caused by inhibitor alone subtracted from percent cell death observed with inhibitor plus MPP^+. Quinidine (0.1 µM) had no effect on cell death without MPP^+. One-way ANOVA and least significant difference *post-hoc test*, $*P < 0.05$, $***P < 0.001$.

Inhibiting CYP2D6 increases 1-methyl-4-phenylpyridinium induced neurotoxicity

Quinidine (**Figure 10A**) significantly enhanced cell death (4 ± 1 to 9 ± 1%) caused by MPP^+ at 10 and 25 μM ($P < 0.05$). To confirm the effects of inhibiting CYP2D6 on MPP^+ neurotoxicity, three other CYP2D6 inhibitors (which are not substrates or inhibitors of CYP3A) were tested (Chauret *et al.*, 2001; Yamamoto *et al.*, 2003). Metoprolol (**Figure 10B**), propranolol (**Figure 10C**) and timolol (**Figure 10D**) significantly increased MPP^+-induced cell death by 8 ± 2 to 22 ± 5% ($P < 0.05$). There was no effect of quinidine or timolol alone on neurotoxicity; however, metoprolol alone increased cell death by 5 ± 2 to 7 ± 1% and propranolol alone increased cell death by 5 ± 5 to 21 ± 3%. The results in **Figure 10** are shown as the difference between percent cell death caused by inhibitor plus MPP^+ and inhibitor alone, where percent cell death observed with inhibitor alone was subtracted from percent cell death observed with inhibitor plus MPP^+.

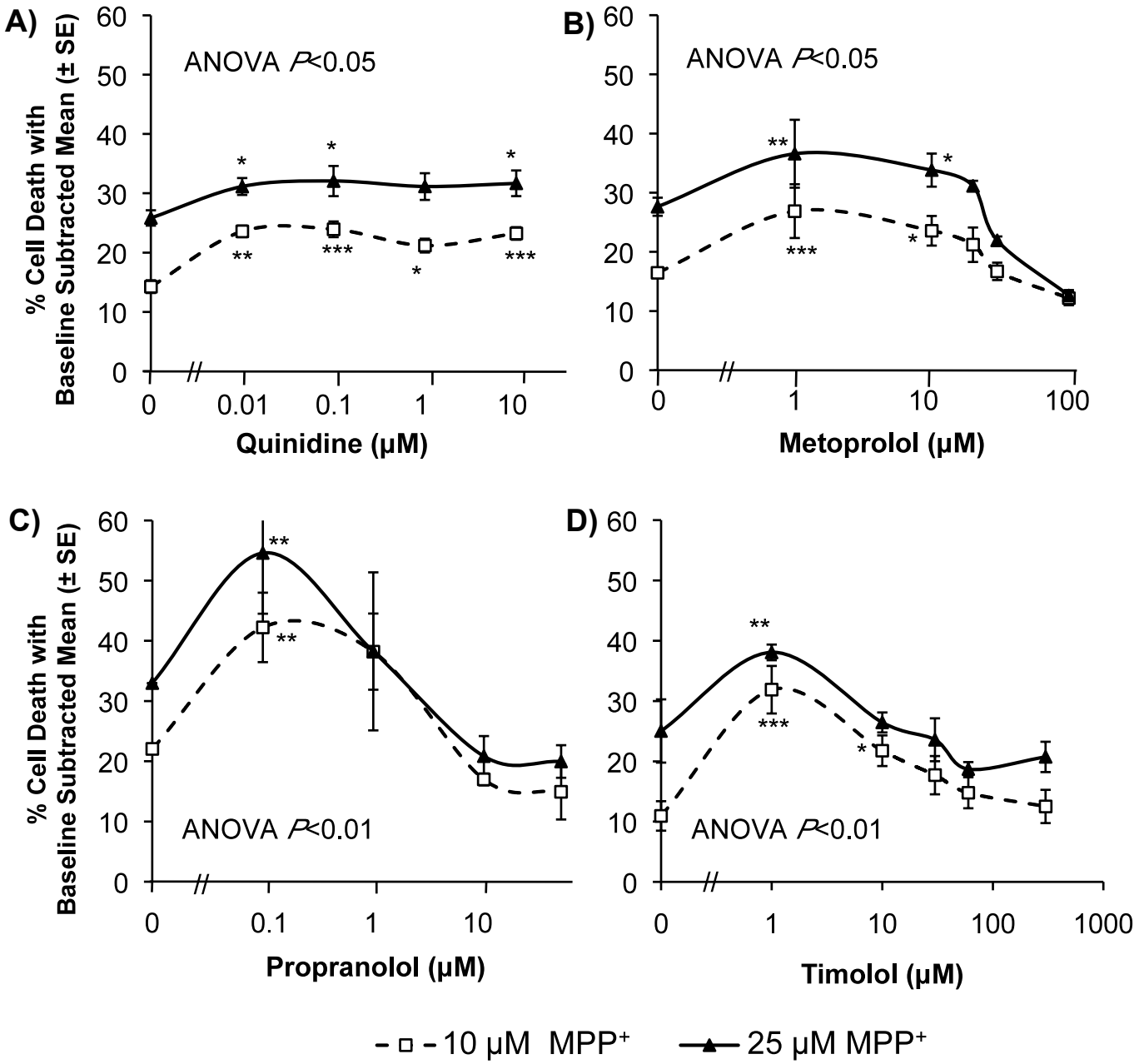

Figure 10: The effect of CYP2D6 inhibition on MPP^+ neurotoxicity.
There was a significant increase in MPP^+ neurotoxicity in the presence of (**A**) quinidine (by 4 ± 1 to 9 ± 1%), (**B**) metoprolol (by 8 ± 2 to 11 ± 6%), (**C**) propranolol (by 20 ± 3 to 22 ± 5%) and (**D**) timolol (by 13 ± 1 to 21 ± 4%). Results are shown as percent cell death caused by inhibitor alone (baseline) subtracted from percent cell death observed with inhibitor plus MPP^+. Quinidine and timolol had no effect on cell death without MPP^+. Metoprolol increased cell death by 7 ± 1% (1 μM) and by 5 ± 2% (10–100 μM) without MPP^+. Propranolol increased cell death by 7 ± 25% (0.1 μM), 5 ± 5% (1 μM), 16 ± 1% (10 μM) and 21 ± 3% (50 μM) without MPP^+. Mean of triplicate wells from two to four experiments ± SEM. One-way ANOVA and least significant difference post-hoc test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Influence of CYP3A and CYP2D6 in 1-methyl-4-phenylpyridinium-induced neurotoxicity

As CYP3A and CYP1A2 can also inactivate neurotoxins (Coleman *et al.*, 1996; Gilham *et al.*, 1997), their effect in our in-vitro MPP^+ neurotoxicity model was tested. **Figure 11A** shows the relative levels of CYP1A (0.26 ± 0.05 pmol), CYP2D6 (0.29 ± 0.03 pmol) and CYP3A (2.0 ± 0.1 pmol) expression / μg of SH-SY5Y whole-cell lysate protein, determined by comparison with their respective cDNA-expressed CYP standard curves. As CYP3A had higher expression than both CYP2D6 and CYP1A, we assessed the influence of CYP3A activity on MPP^+ neurotoxicity by using an inhibitor of CYP3A, ketoconazole (Bourrie *et al.*, 1996). Inhibiting CYP3A significantly (ANOVA, $P < 0.001$) increased cell death by MPP^+ (**Figure 11B**). Low and selective doses of ketoconazole (0.0001–0.1 μM) significantly enhanced MPP^+-induced (10 and 25 μM) cell death by 14 ± 2 to 30 ± 3%. To determine the relative influence of CYP2D6 and CYP3A on MPP^+ neurotoxicity, both inhibitors were administered together or alone at doses close to their corresponding Ki established in human liver microsomes (Bourrie *et al.*, 1996) (**Figure 11C**). Both ketoconazole (6 ± 1%) and quinidine (7 ± 2%) significantly increased MPP^+-induced cell death compared with no inhibitor ($P < 0.01$). Co-treatment with ketoconazole and quinidine showed an additive effect (13 ± 2%) on MPP^+-induced neurotoxicity. Ketoconazole alone, quinidine alone and ketoconazole plus quinidine without MPP^+ had no effect on cell death. There was a significant effect of quinidine ($P < 0.001$) and ketoconazole ($P < 0.001$) on MPP^+ neurotoxicity but no significant interaction between the two drugs (two-way ANOVA, $P = 0.64$, $F = 0.23$). This suggests that both CYP2D6 and CYP3A independently influence MPP^+ neurotoxicity in SH-SY5Y human neuroblastoma cells.

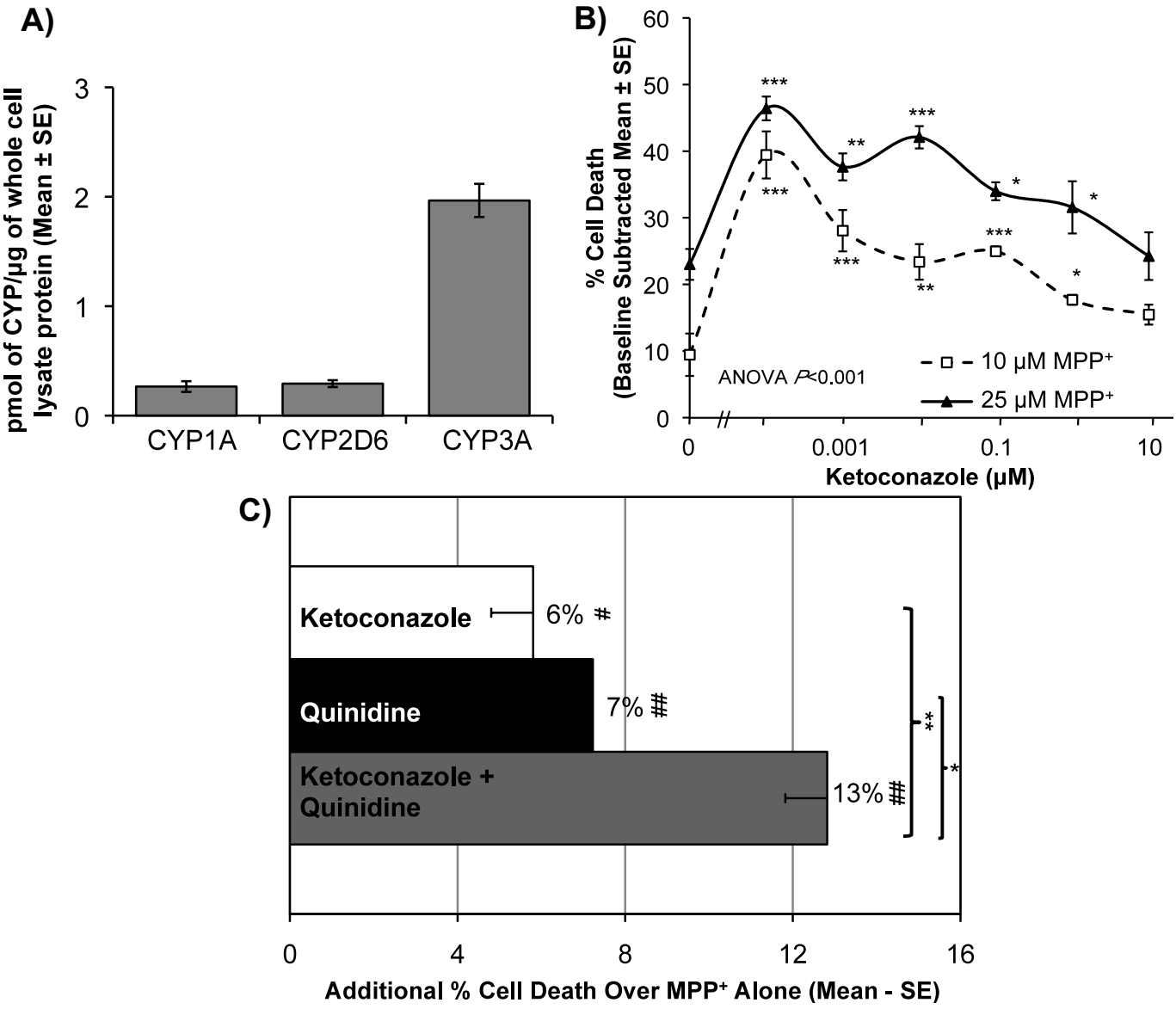

Figure 11: The role of CYP2D6 and CYP3A in MPP^+ neurotoxicity.
(A) SHSY5Y cells express CYP1A, CYP2D6 and CYP3A at 0.26 ± 0.05, 0.29 ± 0.03 and 2.0 ± 0.1 pmol / µg of whole-cell lysate protein, respectively. Mean band density ± SEM from three or four western blots. **(B)** The effects on MPP^+ neurotoxicity of inhibiting CYP3A with ketoconazole. Ketoconazole increased cell death in the presence of MPP^+. Results are shown as percent cell death minus baseline cell death of inhibitor-treated cells with no MPP^+. One-way ANOVA and least significant difference post-hoc test, $*P < 0.01$, $**P < 0.005$, $***P < 0.0001$. **(C)** The effects on MPP^+ (10 µM)-induced cell death of inhibiting CYP3A and CYP2D6, alone and together with their respective specific inhibitors ketoconazole (0.001 µM) and quinidine (0.1 µM). Results are derived from percent cell death caused by inhibitor alone (baseline) subtracted from percent cell death observed with inhibitor plus MPP^+. Ketoconazole alone, quinidine alone and ketoconazole plus quinidine without MPP^+ had no effect on cell death. One-way ANOVA and LSD post-hoc test, $*P < 0.01$, $**P < 0.005$ compared with inhibitor treatments and ##P < 0.01, ###P < 0.005 compared with MPP^+ treatment alone. Mean of triplicate wells from three experiments ± SEM.

Discussion

Here we show for the first time that endogenous CYP2D6 metabolic activity directly influences MPP^+ neurotoxicity in living cells. The detection of enzymatically functional CYP2D6 protein in SH-SY5Ycells confirms data suggesting that these cells contain CYP2D6 assessed by the metabolism of [^{3}H]-codeine to morphine, a pathway mediated primarily by CYP2D6 (Poeaknapo *et al.*, 2004; Boettcher *et al.*, 2005). We showed that quinidine not only increases neurotoxicity caused by MPTP but also that caused by the neurotoxic metabolite MPP^+. More importantly, in addition to quinidine, three other CYP2D6-selective inhibitors (Yamamoto *et al.*, 2003) at low concentrations lead to a significant 9–20% enhancement in cell death caused by MPP^+ alone. This suggests that by inhibiting CYP2D6 we reduced its ability to inactivate this neurotoxin, thereby increasing cell death.

1-Methyl-4-phenylpyridinium can competitively inhibit the metabolism of CYP2D6-specific substrates in liver and brain microsomes (Fonne-Pfister *et al.*, 1987; Jolivalt *et al.*, 1995). Docking studies indicate that the methyl group of MPTP fits into the active site of CYP2D6 (Modi *et al.*, 1997) and CYP2D6 tends to interact with positively charged amines (Yao *et al.*, 2004), suggesting a role for CYP2D6 in the demethylation of both MPTP and MPP^+. Although direct evidence for inactivation of MPP^+ by CYP2D6 is lacking, our study in a human neuronal cell line supports this idea. In agreement with our findings, the CYP2D6 inhibitor fluoxetine (Bertelsen *et al.*, 2003) increased MPP^+ cytotoxicity in PC12 rat pheochromocytoma cells (Han & Lee, 2009). In addition, over-expression of CYP2D6 in PC12 cells was shown to be protective against MPP^+-induced cytotoxicity (Matoh *et al.*, 2003). Together this indicates that CYP2D6 can play a neuroprotective metabolic role against MPTP and MPP^+ neurotoxicity, by increasing inactivation of MPTP and possibly MPP^+. Moreover, higher levels of functional CYP2D6 may be important in protecting individuals from PD-

causing neurotoxins.

Genetically variable CYP2D6 is highly polymorphic (http://www.cypalleles.ki.se/cyp2d6.htm) and has large variability in brain expression, which is probably amplified by environmental inducers/inhibitors of CYP2D6. Liver CYP2D6 is genetically variable but essentially uninducible (Edwards *et al.*, 2003); there is large variability in the expression and activity reported in human liver microsomes, i.e. metabolic inactivation of MPTP by CYP2D6 is shown to range from 0 to 90% (Coleman *et al.*, 1996; Gilham *et al.*, 1997). The large variation in CYP2D6 protein levels (influenced mostly by genetics) may make some individuals more or less prone to the effects of neurotoxins. Individuals who are genetically poor metabolizers of CYP2D6 are at increased risk of developing PD (McCann *et al.*, 1997), especially when exposed to pesticides (Deng *et al.*, 2004; Elbaz *et al.*, 2004). Pesticides such as carbaryl, atrazine, diazinon, chlorpyrifos and parathion can be metabolized by CYP2D6 (Lang *et al.*, 1997; Sams *et al.*, 2000; Hodgson, 2001; Tang *et al.*, 2002). In the brain, where expression of CYP2D6 is relatively high compared with other CYPs [i.e. CYP3A4 and CYP1A2 (Dutheil *et al.*, 2009)], CYP2D6 may play a role in the localized elimination of pesticides. Thus, CYP2D6 may represent a link between genetic and environmental factors that influences the development of PD, especially as this enzyme is ideally localized in brain regions and cells affected in PD. If the degree of toxicity is determined, at least in part, by a balance between activation (i.e. by monoamine oxidase) and inactivation (i.e. by CYP2D6) of neurotoxins (Herraiz *et al.*, 2006), then genetically higher or induced levels of brain CYP2D6 could increase neurotoxin inactivation and compete with neurotoxin activation.

Smokers display a 50% reduced risk of developing PD (Alves *et al.*, 2004). We have shown that smokers have higher brain CYP2D6 than non-smokers (Mann *et al.*, 2008) and these higher CYP2D6 levels may be partially responsible for the protection against PD

observed in smokers. Nicotine, a component of cigarette smoke, has been shown to be neuroprotective (O'Neill *et al.*, 2002; Quik *et al.*, 2006b; Singh *et al.*, 2009) and it induces brain CYP2D in mice (Singh *et al.*, 2009), rat (Yue *et al.*, 2008) and monkey (Mann *et al.*, 2008) with no change in liver CYP2D. In fact, similar to smokers, nicotine significantly increases CYP2D in regions affected in PD like the substantia nigra and striatum (Mann *et al.*, 2008; Singh *et al.*, 2009). Collectively, these data suggest that nicotine in cigarette smoke may be responsible for a smoker's decreased risk of developing PD, perhaps in part through inducing brain, but not liver, CYP2D6.

Recently, in an MPTP-induced PD mouse model, nicotine was shown to reduce MPP^+ levels in the striatum (Singh *et al.*, 2009), which is consistent with an induction by nicotine of striatal CYP2D with a subsequent increase in MPTP and/or MPP^+ deactivation. Nicotine was also shown to cause a recovery of CYP2D mRNA and activity that had been lost with MPTP treatment, probably through the induction of CYP2D. The ability of nicotine to induce brain CYP2D may be of benefit in PD, reducing further neuronal damage from toxins. It is also possible that higher levels of CYP2D may assist in maintaining dopaminergic tone as CYP2D can synthesize dopamine (Hiroi *et al.*, 1998; Bromek *et al.*, 2010), although an effect of CYP2D activity on dopamine levels in vivo has not yet been demonstrated. The ability to alter brain CYP2D6 expression may be important, especially as hepatic CYP2D6 is generally uninducible (Edwards *et al.*, 2003). Given the punctate region- and cell-specific expression of CYP2D6, small changes in expression may have a large effect in localized toxin inactivation and neurotoxicity.

Consistent with higher CYP2D6 being protective (Matoh *et al.*, 2003), our current study showed that inhibiting CYP2D6 enhanced MPP^+ neurotoxicity. Quinidine at low concentrations was less effective at increasing MPP^+-induced neurotoxicity than the other three

CYP2D6 inhibitors, despite observing complete inhibition of AMMC metabolism in SH-SY5Y cell membranes with 1 μM quinidine. This lower than expected effect of quinidine on MPP^+ neurotoxicity may be due to the removal of quinidine by CYP3A as it is a substrate for CYP3A4 (Nielsen *et al.*, 1999). It may also be due to the ability of quinidine to potently inhibit transporter systems such as Oct-3 (extraneuronal monamine transporter) (Liou *et al.*, 2007) and vesicular monoamine transporter (Staal *et al.*, 2001) that transport MPP^+. Transporter expression is not well characterized in SH-SY5Y cells but these cells do express vesicular monoamine transporter (Fong *et al.*, 2007). Therefore, quinidine may compete with some transporters of MPP^+, preventing influx and reducing the toxic effects of MPP^+, thereby attenuating the effect of quinidine on increasing neurotoxicity through inhibition of CYP2D6 MPP^+ inactivation. Alternatively, the reduced MPP^+ neurotoxicity at high concentrations of timolol, propranolol and metoprolol (β-blockers) may be through the blockade of Na^+ and Ca^{++} channels and/or via adrenergic receptors (Goto *et al.*, 2002). Timolol and other β-adrenergic antagonists have been shown to be neuroprotective against glutamate-induced toxicity in retinal ganglion cells (Goto *et al.*, 2002; Wood *et al.*, 2003).

The SH-SY5Y cells are very useful for modeling PD in vitro (Presgraves *et al.*, 2004; McMillan *et al.*, 2007); however, they do not entirely reflect the intact brain and its variable composition of activating (i.e. monoamine oxidase) and inactivating (i.e. CYP2D6) enzymes. In these cells, we found higher CYP3A expression than CYP2D6, whereas in the brain, the expression and activity of CYP3A are much lower than CYP2D6 and below detection in some regions (Voirol *et al.*, 2000; Dutheil *et al.*, 2009). A direct comparison of the inhibition of MPTP (50 μM) metabolic inactivation using quinidine (CYP2D6), ketoconazole (CYP3A) or furafylline (CYP1A2) in nine human CYP2D6 extensive metabolizer livers indicated that CYP2D6 was the main enzyme involved (54%), with minor contributions from CYP1A2

(32%) and CYP3A4 (12%) (Coleman *et al.*, 1996); the relative contribution of CYP2D6 and CYP1A2 was subsequently replicated (Gilham *et al.*, 1997). These data are consistent with the higher affinity of yeast-expressed CYP2D6 (K_m = 39 μM) compared with CYP1A2 (K_m = 2.2 mM) for MPTP (Coleman *et al.*, 1996). At substantially higher concentrations of MPTP (2 mM), CYP1A2 and CYP3A4 contributed 49 and 25% of the inactivation, respectively (Coleman *et al.*, 1996) suggesting that, like CYP1A2, CYP3A4 may have a lower affinity for MPTP than CYP2D6. Although these studies investigated MPTP inactivation, the same relative enzymatic contributions to MPP^+ inactivation may occur given the structural resemblance of the two substrates; this, however, has not yet been demonstrated. Thus, compared with our results in SH-SY5Y cells, the impact of CYP2D6 may be much greater in the human brain compared with CYP3A due to the higher brain expression of CYP2D6 and its potential for higher rates of neurotoxin metabolic inactivation.

In conclusion, SH-SY5Y cells express enzymatically functional CYP2D6. Upon inhibiting this enzyme there is a 9–20% enhancement of MPP^+-induced neurotoxicity, suggesting that this enzyme contributes importantly to protection against PD-causing neurotoxins. In addition, because nicotine treatment can increase CYP2D6 in the brain, this offers a potential therapeutic approach to protecting the brain from neurotoxins that are also substrates of CYP2D6.

Acknowledgements

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Significance of chapter

We confirmed that the SH-SY5Y dopaminergic cell line expresses functional CYP2D6. To determine the role of CYP2D6 in neuroprotection we utilized the intrinsic expression of CYP2D6 in SH-SY5Y cells and inhibited CYP2D6 functional ability to inactivate the neurotoxin MPP^+. MPP^+, the potent neurotoxic metabolite of MPTP, was used to avoid the need for MAO-B activation from MPTP to MPP^+ in our SH-SY5Y cell culture model. We demonstrated that inhibiting CYP2D6 augmented neurotoxicity by MPP^+, using four distinct inhibitors of CYP2D6. This study supports the theory that functional CYP2D6 in the CNS is involved in neuroprotection, likely by inactivating neurotoxins. It also suggests that functional inhibition of brain CYP2D6 by xenobiotics, or changes in functional levels of CYP2D6 protein (i.e., by inducers), may lead to altered neurotoxicity by toxins metabolized by CYP2D6 and therefore altered risk for PD.

Chapter 2: Induction of the drug metabolizing enzyme CYP2D in monkey brain by chronic nicotine treatment

Amandeep Mann, Sharon L. Miksys, Anna Lee, Deborah C. Mash, and Rachel F. Tyndale

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Dr. Rachel F. Tyndale and Dr. Sharon L. Miksys designed the monkey study and contributed to manuscript editing. Amandeep Mann (1) optimized the assay for detecting brain CYP2D in monkey, (2) conducted the western blotting to measure changes in brain CYP2D between saline and nicotine treated monkeys, (3) preformed the data analysis and (4) wrote the manuscript. Dr. Sharon L. Miksys conducted the immunohistochemistry and the western blotting to assess difference in brain CYP2D6 between human smokers and non-smokers. Anna Lee conducted the western blotting to measure difference in hepatic CYP2D between nicotine and saline treated monkeys. Dr. Deborah Mash provided the human samples. Ewa Hoffmann conducted the genotyping of the human samples. All authours edited the manuscript.

Abstract

Cytochrome P450 (CYP) 2D6, an enzyme found in the liver and the brain, is involved in the metabolism of numerous centrally acting drugs (i.e. antidepressants, neuroleptics, opiates), endogenous neurochemicals (i.e. catecholamines) and in the inactivation of neurotoxins (i.e. 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.

Introduction

Cytochrome P450 2D6 (CYP2D6) is an enzyme that metabolizes a range of endogenous (i.e. dopamine) and exogenous (i.e. parathion) substrates. It metabolizes approximately 25% of all clinically used drugs including analgesics (i.e. codeine), neuroleptics (i.e. clozapine), antidepressants (i.e. imipramine), and drugs of abuse (i.e. 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.*, 2000b; 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.*, 1991), readily metabolize CYP2D6 probe drugs (i.e. dextromethorphan), indicating a functional enzyme. Furthermore, in rat brain both mRNA and protein correlate with activity (Miksys *et al.*, 2000b). 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.*, 2003d) 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; Quik *et al.*, 2006b). It also reduces, and maintains the reduction in, the dyskinetic side effects of levodopa (Quik *et al.*, 2007b). 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.

Materials and methods

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 antimouse 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).

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 6**). 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.

Table 6: **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	9.8	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	21.8	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

[a] Unknown 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 (hr); Pk/d: packs/day; Cig/d: cigarettes/day.

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 & 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.*, 2006a). 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 ^{o}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 Immunocytochemical (ICC) CYP2D analysis was performed on frozen sections of fixed tissue as previously described (Lee *et al.*, 2006a). 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 ^{o}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 antirabbit 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.

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 & Bowden, 2000) and rhesus monkey **(**Macaca mulatta**)** (Snider, 1961) as previously described (Joshi & Tyndale, 2006b; Lee *et al.*, 2006a). 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 3000*g* for 10 min at 4 ^{o}C. The supernatant was collected and the pellet resuspended and centrifuged again at 3000*g* for 10 min at 4 ^{o}C. The combined supernatant was centrifuged at 110 000*g* for 90 min at 4 ^{o}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 ^{o}C. The protein content of brain membranes was assayed using the Bradford technique with a Bio-Rad Protein Assay kit.

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 ^{o}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 sec –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 ^{o}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 ^{o}C prior to probing blots.

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 2x S.D., and removed from the analysis.

Results

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 (**Figure. 12**), 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.

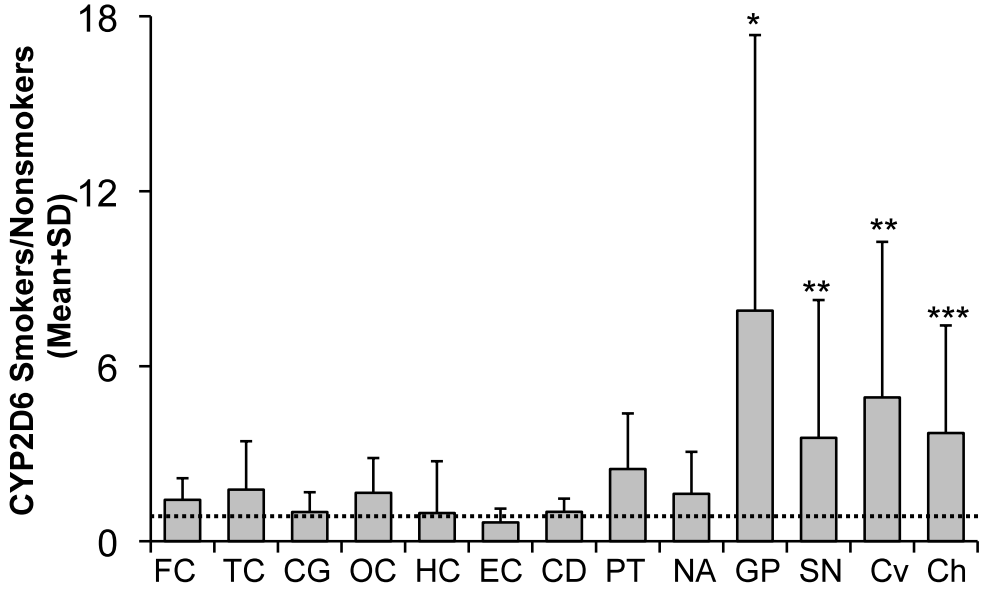

Figure 12: Human CYP2D6 levels are higher in some brain regions of smokers compared to non-smokers.
The dotted line represents the relative level of brain CYP2D6 in nonsmokers. There are significantly higher levels of CYP2D6 in smokers (n =10) relative to non-smokers (n = 3–16) in the globus pallidus, 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.

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 (**Figure 13A**). 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 (**Figure 13B**). Monkey liver purified PAb detected the same two bands (**Figure 13C right**) as those detected by the unpurified antibody (**Figure 13C 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 (**Figure 13C centre**), indicating the specificity of the PAb for CYP2D6. The lower of the two bands co-migrated with monkey liver and cDNA-expressed CYP2D6 (**Figure 13B**). 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 (two-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 **Figure 13B** and these dilution curves were used to determine the linear range of detection for the assay (**Figure 13D**). 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.

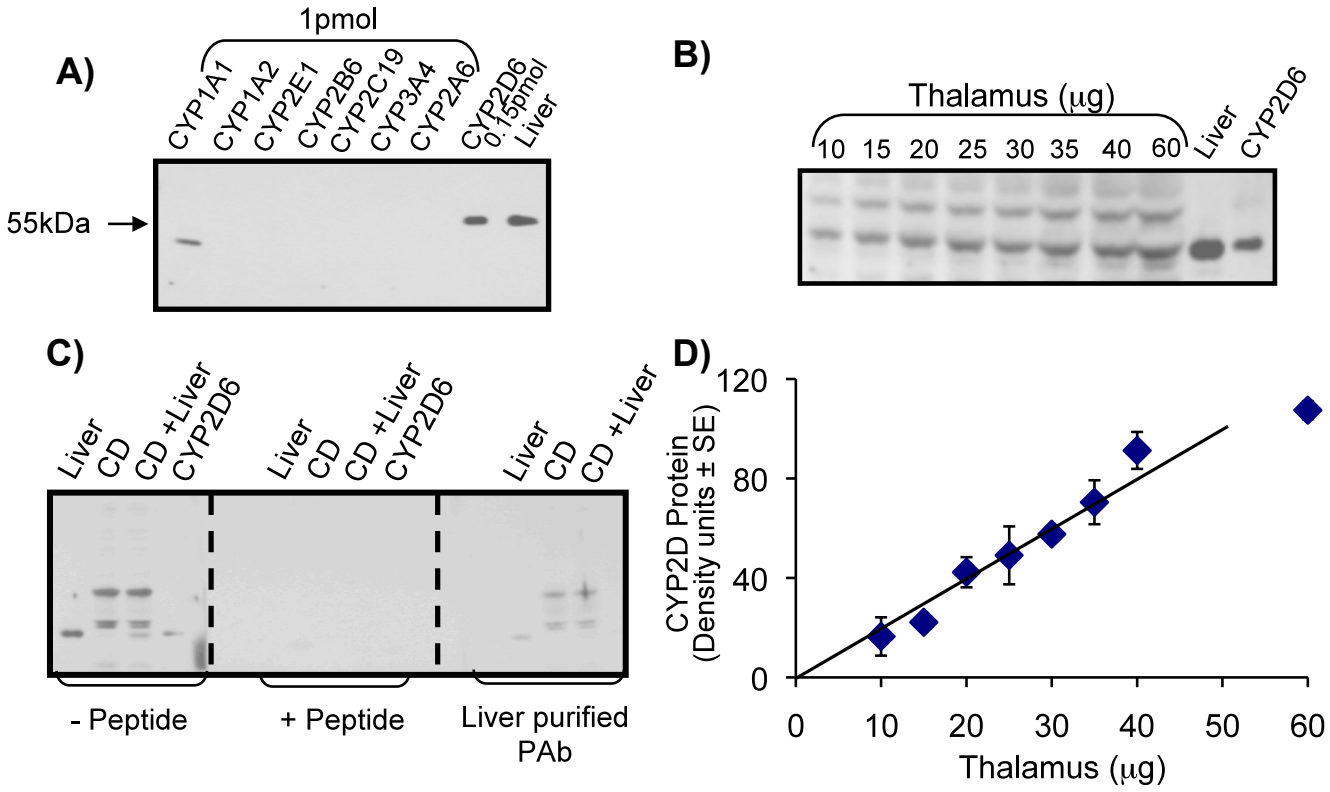

Figure 13: CYP2D is detectable and quantifiable in monkey brain
(A) A western blot testing the cross-reactivity of the PAb with cDNA-expressed CYPs. CYPs were loaded at 1 pmol, except CYP2D6 which was loaded at 0.15 pmol (7 times lower concentration than other CYPs); monkey liver was loaded at 5 μg. **(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).

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 (**Figure 14**). 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 7** and **Figure 15**). Specific cells in the hippocampus CA3 polymorphic layer lining the cerebral aquaduct, in the thalamus and in the pontine nucleus, showed moderate staining for CYP2D compared with the surrounding cells and tissue (**Figure 15**). There was also some staining in Purkinje cells and in glial cells in the white matter of the cerebellum (**Table 7 and Figure 18D,F**). Regions such as the hippocampus, which displayed very low levels of basal expression using western blotting (**Figure 14**), showed moderate staining in some neurons using ICC (**Figure 15A**), 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 (**Figure 15E**).

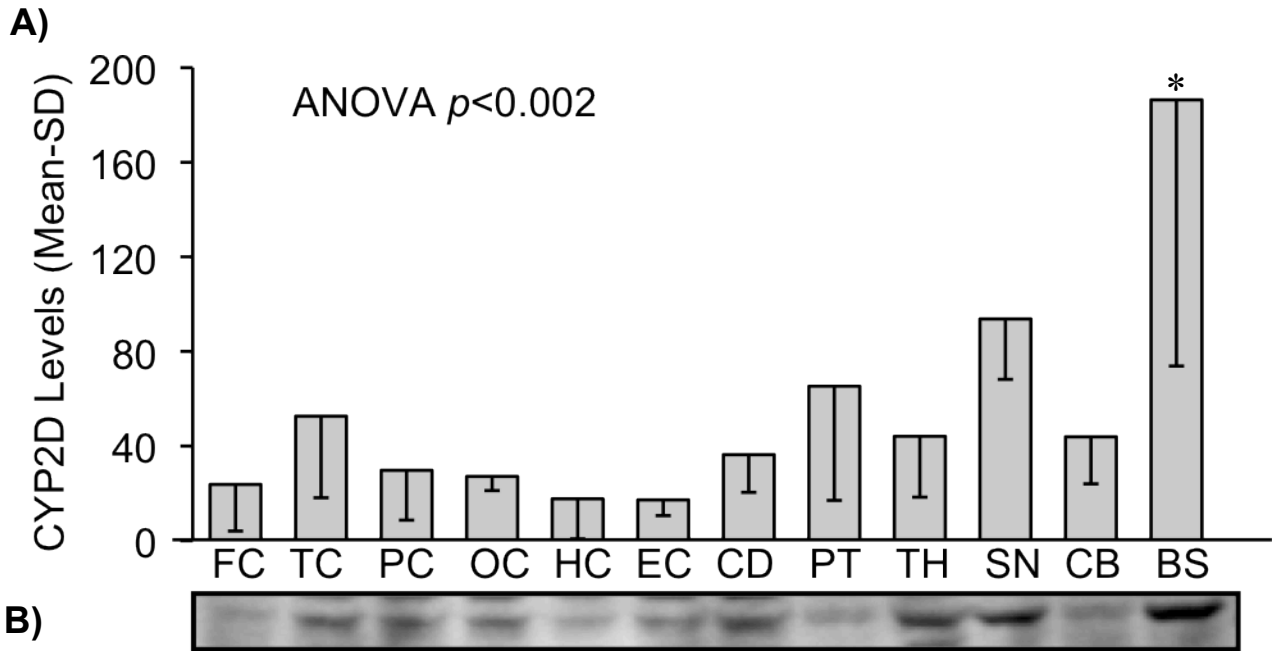

Figure 14: Significant variation in expression of monkey brain CYP2D across 12 brain regions in saline-treated monkeys

(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 μg 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

Table 7: 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 posteriolateral nucleus	++	++
Cerebellum		
molecular layer	+	+
Purkinje cells	+	++++
granule cell layer	+	+
white matter	-	-/+
cerebellar nuclei neurons	+	+++
Brain Stem		
motor nuclei neurons	++	++
aquaduct	+++	+++
Lateral geniculate nucleus	++	++

++++ intense, +++strong, ++moderate, +weak, - no staining

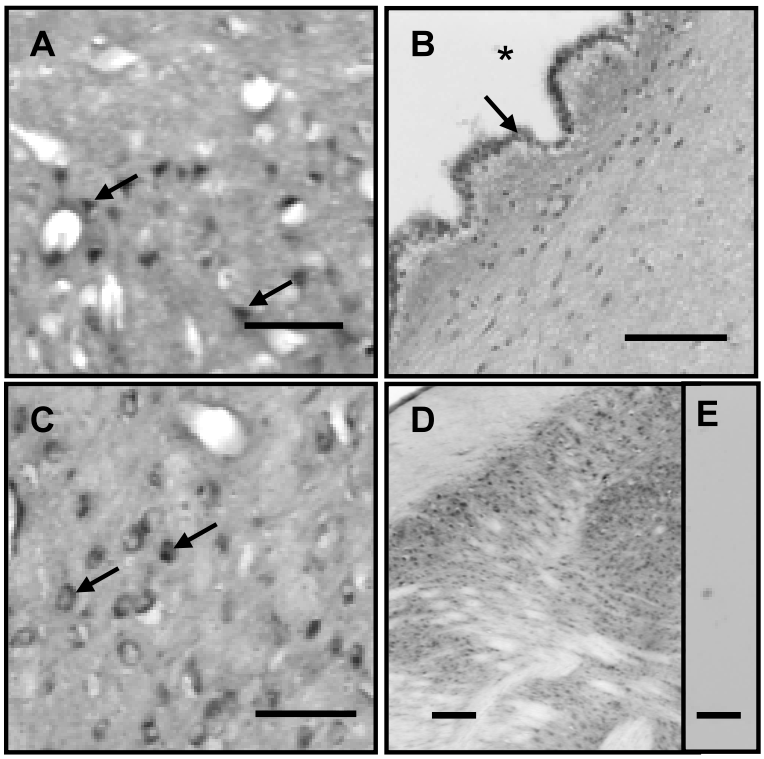

Figure 15: Region- and cell-specific expression of CYP2D in saline-treated monkeys
(A) There was intense immunoreactivity seen in neurons (arrows) in the CA3 polymorphic layer in the hippocampus. **(B)** Cells lining the cerebral aquaduct (*) intensely stained for CYP2D. **(C)** Neurons in the thalamus (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 μm.

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 (two-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 (**Figure 16**). 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 7** and **Figure 17**). Although the frontal cortex and cerebellum displayed no change in CYP2D levels with chronic nicotine treatment when assayed by western blotting (**Figure 16**), the intensity of staining for CYP2D was higher in specific cells such as pyramidal neurons in layers III–V of the frontal cortex (**Table 7** and **Figure 18B,C**), and Purkinje cells and neurons in the deep cerebellar nuclei of the cerebellum (**Table 7** and **Figure 18D–G**) in nicotine-treated animals.

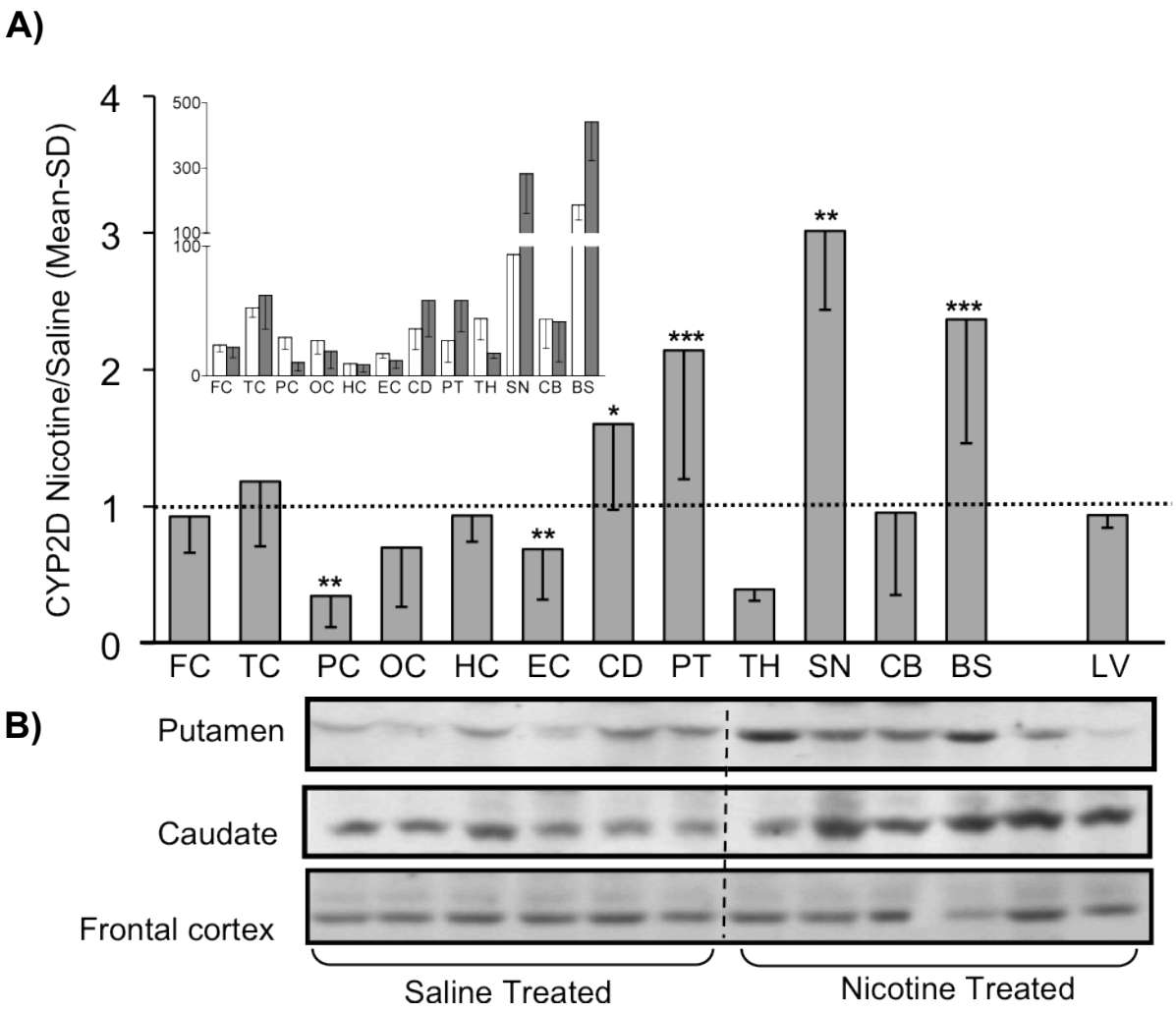

Figure 16: Induction of monkey brain CYP2D following chronic nicotine treatment
(A) A bar graph representing relative change in each region upon nicotine treatment. Mean levels-SD of CYP2D in nicotine-treated monkeys relative to the mean levels in saline treated monkeys. The dotted line at 1 represents 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 Figure 3 for brain region abbreviations; **P*=0.07, ***P*< 0.05, ****P*=0.001.

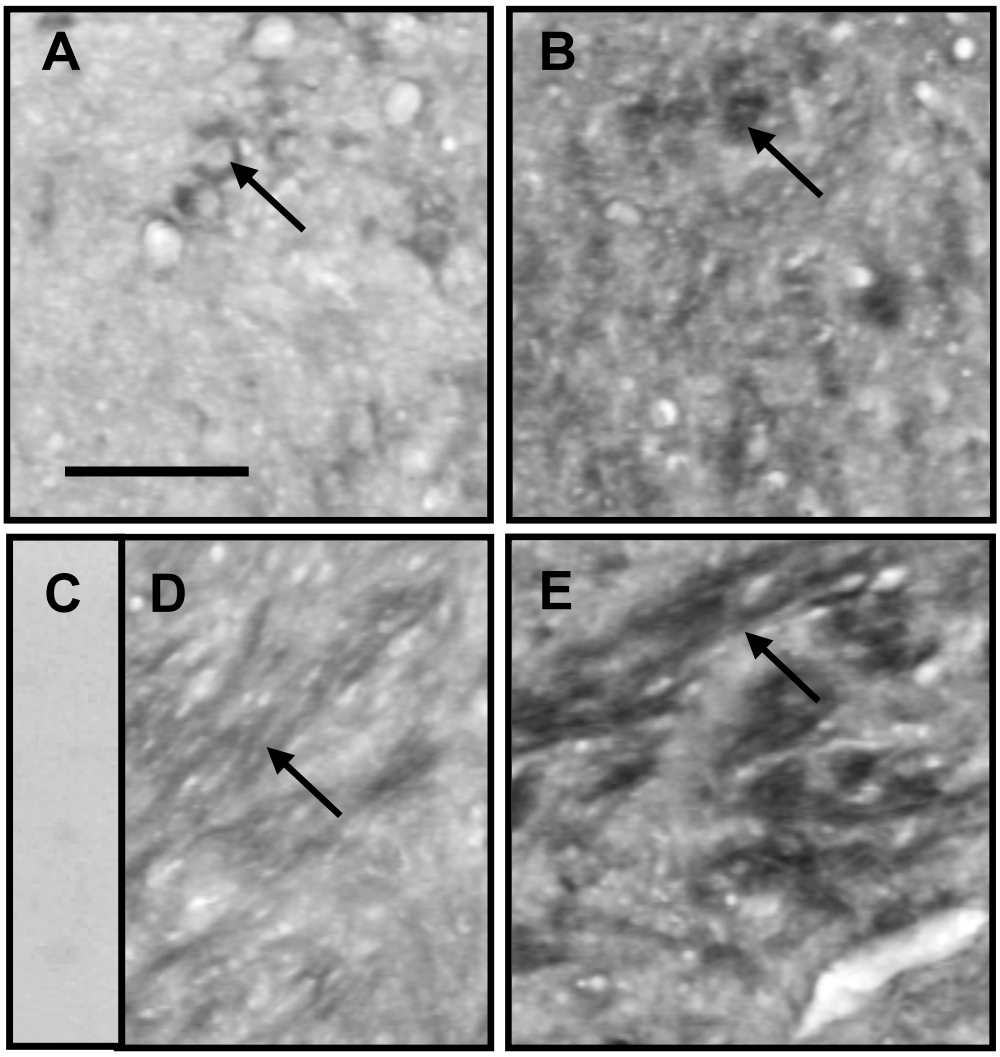

Figure 17: Higher CYP2D immunostaining in substantia nigra of nicotine-treated animals

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 μm, for A–E.

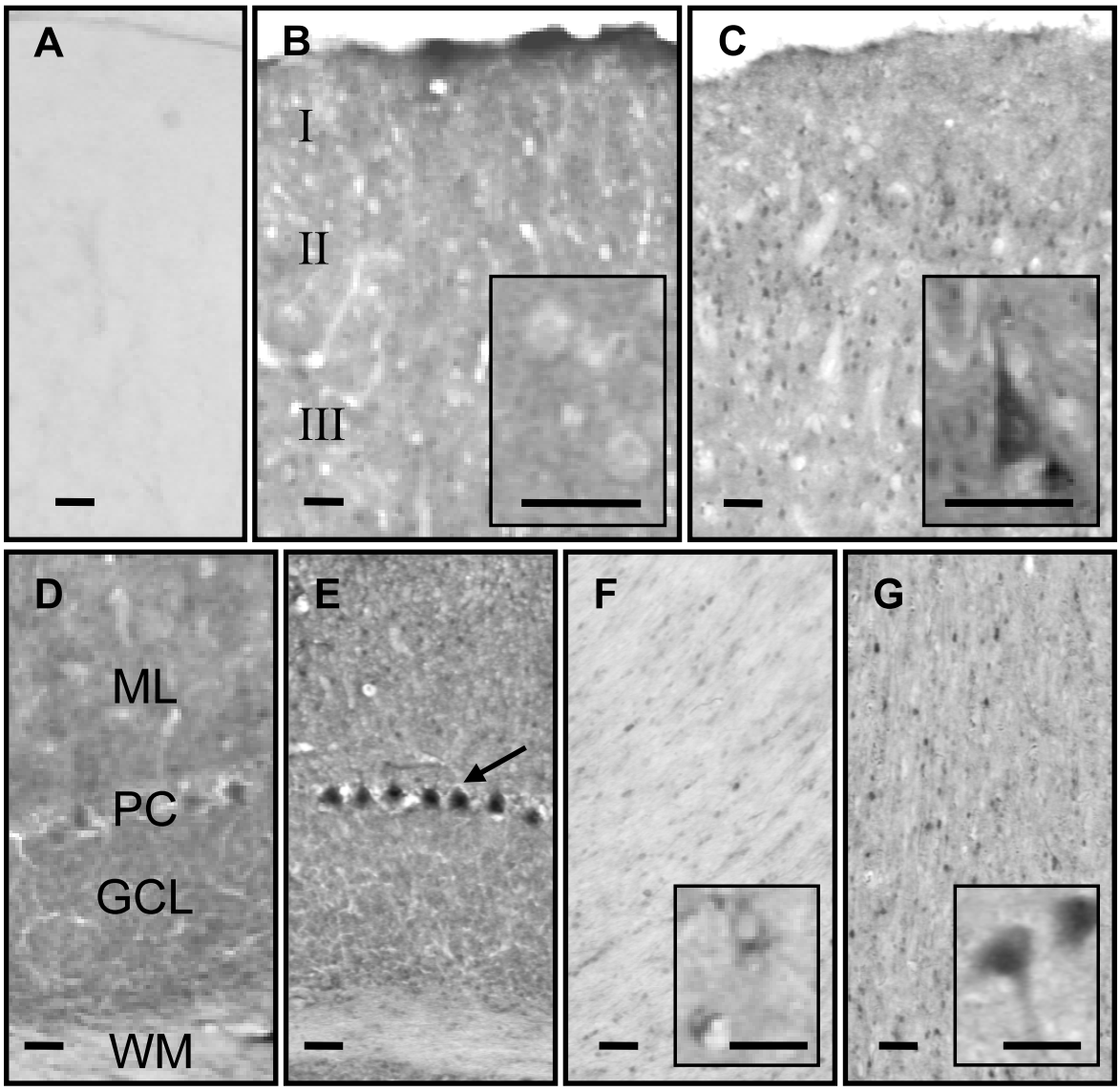

Figure 18: Higher CYP2D immunostaining in frontal cortex and cerebellum of nicotine-treated animals

(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 μm.

Discussion

This is the first study to show the induction of CYP2D in a nonhuman 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 (i.e. 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 nonhuman 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 (i.e. MPTP), and in PD models monkeys exhibit similar Parkinsonian symptoms to humans (Quik *et al.*, 2006a; Quik *et al.*, 2006b; Quik *et al.*, 2007b).

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; Riedl *et al.*, 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 (i.e. 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 & Wedlund, 1992). There is evidence of very poor correlation between drug effect and plasma levels for neuroleptics and antidepressants (Michels & Marzuk, 1993a). 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 selective 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 isoforms have previously been identified in rodent (i.e. 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 (i.e. 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 (i.e. pyramidal neurons in frontal cortex) (Norris *et al.*, 1996; Miksys *et al.*, 2000b; 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.*, 2006a) and CYP2E1 (Joshi & Tyndale, 2006b) 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 posttranscriptional 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.

Acknowledgments

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Significance of chapter

This was the first study to examine the constitutive expression of CYP2D6 protein in the monkey brain. The expression was observed to be brain region and cell specific (i.e., cerebellar Purkinje cells). Furthermore, we showed that CYP2D6, which is considered uninducible in the liver, could be induced in the brain in a region- and cell-specific manner by nicotine. Similarly, smokers had significantly higher brain CYP2D6 levels in select brain regions, including the SN, compared to non-smokers. The high expression of CYP2D6 and inducibility by nicotine in the basal ganglia and the SN could be of importance in PD as these brain structures are directly affected. Both the ideal localization, and induction of CYP2D6 in these regions, may increase local inactivation of endogenous and/or exogenous neurotoxins reducing CNS damage. Increased CYP2D6 levels in the brain may be one mechanism of neuroprotection elicited by smoking and nicotine. Overall, changes in brain CYP2D6 expression in select-regions and -cell types may influence localized substrate metabolism affecting neurotoxin inactivation, metabolism of neurosteroids and neurotransmitters, and the levels of CNS acting drugs.

Chapter 3: The neuroprotective enzyme CYP2D6 increases in the brain with age and is lower in Parkinson's disease patients.

Amandeep Mann, Sharon L. Miksys, Andrea Gaedigk, Stephen J. Kish, Deborah C. Mash, and Rachel F. Tyndale

Dr. Rachel F. Tyndale, Dr. Sharon L. Miksys and Amandeep Mann designed the study. Amandeep Mann (1) optimized the assay to specifically detect human brain CYP2D6, (2) conducted the western blotting to measure changes in brain CYP2D6 with age and difference in CYP2D6 between PD cases and controls, (3) optimized and performed fluorescent immunocytochemistry in PD cases and controls, (4) preformed the data analysis and (5) wrote the manuscript with assistance from Dr. Rachel F. Tyndale. Dr. Sharon L. Miksys conducted the western blotting for some of the age samples and edited the manuscript. Dr. Andrea Gaedigk provided fetal samples for the ontogeny study and conducted the extensive genotyping for all samples used in this study. Dr. Stephen Kish provided samples used in the ontogeny study. Dr. Deborah Mash provided samples of PD cases and age-matched control. All authours edited the manuscript.

Abstract:

CYP2D6 is a drug metabolizing enzyme expressed in the brain that also metabolizes endogenous neural compounds (i.e. catecholamines) and inactivates neurotoxins (i.e. MPTP, TIQ). Genetically poor *CYP2D6* metabolizers are at higher risk for developing Parkinson's disease (PD), a risk that increases with exposure to pesticides. As age is a risk factor for PD we measured the ontogenic expression of CYP2D6 in human brain, and compared brain CYP2D6 levels in PD cases to age-matched controls. CYP2D6 increased from fetal to 80 years of age (n=76), exhibiting three distinct phases of change. Compared to PD controls, PD cases had ~40% lower CYP2D6 levels in the frontal cortex, cerebellum, and the hippocampus, even when controlling for *CYP2D6* genotype. In contrast, CYP2D6 levels in cases were similar to controls in PD-affected brain areas, the substantia nigra and caudate, consistent with higher astrocytic and cellular CYP2D6 staining observed in PD cases. In summary, the lower CYP2D6 levels in PD cases may have reduced their ability to inactivate PD-causing neurotoxins contributing to their disease risk.

Introduction:

Parkinson's disease (PD) is a neurodegenerative disorder with a poorly characterized etiopathology. While aging is a substantial risk factor for developing PD (Lang & Lozano, 1998; Grosset G. D., 2009), individuals that are either genetically predisposed and/or exposed to endogenous or environmental neurotoxins may have accelerated CNS damage leading to PD. It has been proposed that individuals with genetic variants in the *CYP2D6* gene, encoding a non-functional enzyme, have an increased risk for PD which is further increased with exposure to pesticides (McCann *et al.*, 1997; Deng *et al.*, 2004; Elbaz *et al.*, 2004).

CYP2D6 can inactivate neurotoxins such as MPTP (Modi *et al.*, 1997), TIQ (Suzuki *et al.*, 1992), and β-carbolines (Yu *et al.*, 2003d). Furthermore, CYP2D6 can metabolize neurotransmitters including dopamine and serotonin, plus a variety of CNS acting drugs including antidepressants, analgesics, antipsychotics (Wang *et al.*, 2009). Importantly, functional CYP2D6 is expressed in the brain notably within the basal ganglia, a primary area affected in PD (Tyndale *et al.*, 1999; Mann *et al.*, 2008; Bromek *et al.*). Altering CYP2D6 expression and activity in neurological cell lines can influence MPP^+ neurotoxicity; high levels of CYP2D6 are neuroprotective while low levels reduce the neuroprotection (Matoh *et al.*, 2003; Mann & Tyndale). Thus CYP2D6 may play an important role in localized CNS toxin inactivation, which is of particular relevance in PD.

CYP2D6 is genetically polymorphic resulting in large interindividual variability in expression. Brain CYP2D6 protein levels are further influenced by environmental factors (i.e. smoking) unlike CYP2D6 in the liver (Mann *et al.*, 2008; De Gregori *et al.*). This variability may affect susceptibility of individuals to environmental neurotoxins altering their risk for developing PD. Here we genotyped for multiple *CYP2D6* alleles, measured the ontogenic

development of CYP2D6 in the frontal cortex, and determined brain CYP2D6 protein levels in PD cases and age-matched controls.

Methods:

Human tissues

Whole fetal brains (n=18; -226 to -163 days gestational age) were received from the University of Maryland Brain and Tissue Bank of Developmental Disorders (Baltimore, MD). Samples of neonatal to adult frontal cortex (n=58) were obtained from the CAMH (Toronto, ON) and the Brain Endowment Bank, University of Miami (Miami, FL). PD cases and age matched PD controls (n=12/group) were also obtained from the Brain Endowment Bank. PD samples were selected based on neuropathological diagnosis of PD based on the UK PD Society Brain Bank diagnostic criteria and severity of the Hoehn and Yahr scale (Hoehn & Yahr, 1967; Hughes *et al.*, 1992). Controls were selected based on age and no history or neuropathological diagnosis of neurologic or psychiatric disease. Agonal state questionnaire provided information about the events 48 hours prior to death and was used to exclude cases based on prolonged periods of hypoxia or anoxia. **Table 8** summarizes the demographics of all samples used in this study (n=88). This study was approved by the ethics review boards of all four institutions.

Table 8: Demographics of tissue donors used to assess the ontogeny of CYP2D6 and of PD cases and PD controls.

Ontogeny Samples				
Age at death	**N**		**Mean (range)**	**P Value[a]**
Fetal	18		84.6 (54-117 days)	
Postnatal				
0-0.9 years	22		0.32 (0-0.92 years)	0.001
1-19 years	13		7.5 (1.5-18 years)	
20-78 years	23		40.1 (21-78 years)	
Postmortem Interval			**Mean (range)**	**P Value[a]**
Fetal			Unknown	
Postnatal				
0-0.9 years			12.9 (3-26 hours)	0.19
1-19 years			12.9 (6-27 hours)	
20-78 years			15.5 (5-29 hours)	
Gender	**Male**	**Female**	**Unknown**	**P Value[b]**
Fetal	6 (33%)	3 (17%)	9 (50%)	1.0
Postnatal				
0-0.9 years	13 (59%)	9 (41%)		0.25
1-19 years	9 (69%)	4 (21%)		1.0
20-78 years	19 (83%)	4 (17%)		0.16
***CYP2D6* genotype**	Null/Null, Slow/Slow (Activity score 0-1)[c]	Wt/Null, Wt/Slow[d] (Activity score 1-1.5)[c]	Wt/Wt[e] (Activity score 2)[c]	**P Value[f]**
Fetal	8 (62%)	4 (31%)	6 (46%)	0.34
Postnatal				
0-0.9 years	6 (27%)	11 (50%)	5 (23%)	0.59
1-19 years	1 (8%)	7 (54%)	5 (38%)	0.19
20-78 years	3 (13%)	15 (65%)	5 (22%)	0.73

Parkinson's Disease Cases & Controls				
		PD Controls (N=12)	**PD Cases (N=12)**	**P value**
Age	Onset		67 (54-77 y)	
	Death	69 (53-88 y)	75 (53-83 y)	0.23[g]
Gender	Male	7 (58%)	9 (75%)	0.33[h]
	Female	5 (41%)	3 (25%)	
Ethnicity	Caucasian	11 (92%)	12 (100%)	1.0[h]
	Hispanic	1 (8%)	0 (0%)	
Smoking	Non-Smoker	7 (58%)	9 (75%)	0.33[h]
	Smoker	5 (41%)	3 (25%)	
CYP2D6 Genotype	Null/Null, Slow/Slow (Activity score 0-1)[c]	1 (8%)	4 (33%)	0.07
	Wt/Null, Wt/Slow (Activity score 1-1.5)[c]	8 (67%)	5 (42%)	0.60
	Wt/Wt (Activity score 2)[c]	3 (25%)	3 (25%)	1.0

Table 8 values are presented as Mean (range) or N (% of total N)

a. One way ANOVA between age groups
b. Two tailed Fisher's exact test comparing males and females in each age group vs. the total remaining males and females
c. Predicted CYP2D6 activity score based on genotype (Gaedigk *et al.*, 2008); *CYP2D6* allele grouping: Wt, wild-type (**1, *2*); Slow (**9, *10, *17, *29, *41*); Null (**3, *4, *5, *6*)
d. Included in group *A* to determine genotype impact on ontogenic expression of CYP2D6
e. Included in group *A* and *B* to determine genotype impact on ontogenic expression of CYP2D6
f. Two tailed Fisher's exact test comparing variant and wild-type alleles in each age group vs. the total remaining variant and wild-type alleles
g. Two-tailed Student's *t*-test
h. Two-tailed Fisher's exact test

CYP2D6 genotyping

CYP2D6 genotyping was performed as described previously (Gaedigk *et al.*, 2007; Gaedigk *et al.*, 2008; Gaedigk *et al.* 2010). Briefly, a 6.6 kb long-range (XL) fragment was amplified with *CYP2D6*-specific primers and used as a template to detect SNPs or small indels in *CYP2D6*2, *3, *4, *6, *9, *10, *17, *29,* and **41* using TaqMan assays (Applied Biosystems). The *CYP2D6*5* gene deletion, gene duplications/multiplications and *CYP2D7/2D6* hybrid genes were assayed by XL-PCR (Gaedigk *et al.* 2010). The presence of a single *CYP2D6*36* was detected on genomic DNA using allele specific amplification (Gaedigk *et al.*, 2006).

Western blotting

Human brain membranes (80 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoreactive CYP2D6 protein was measured using a rabbit polyclonal CYP2D6 antibody (PAb) diluted 1:3000 (Daiichi Pure Chemicals Co., LTD, Tokyo Japan) followed by peroxidase-conjugated anti-rabbit secondary antibody diluted 1:5000 (Thermo Fisher Scientific, Nepean ON). Equal loading was determined using a monoclonal antibody for actin diluted 1:1000 (Developmental Studies Hydridoma Bank, University of Iowa). CYP2D6 protein was then detected using chemiluminescence and exposed to autoradiographic film for 1 – 3 min. MCID Elite imaging software (Interfocus Imaging Ltd., Linton, U.K.) was used to analyze the data.

Western blotting was repeated 3-5 times with a coefficient of variation <20% between blots. The basal distribution was determined by running the 5 brain regions on a single gel for each PD control (N=6). To detect differences between brain CYP2D6 in PD controls (N=9) and PD cases (N=9), both groups were run on the same gel for each brain region. Data was expressed as the mean of 9 PD controls and 9 PD cases with their respective S.D.

Fluorescent immunocytochemistry

To investigate the presence and impact of gliosis on CYP2D6 staining in an early stage and late stage affected brain region, caudate/putamen and frontal cortex tissues from three PD cases and three age-matched PD controls were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and cyroprotected in 20% sucrose buffer. Frozen 16 mm thin cryostat sections were collected in 10 mM phosphate-buffered saline (PBS) and the sections were treated with 0.3% v/v H_2O_2 for 30 min to remove any endogenous peroxidase activity. Free-floating sections were blocked with 3% w/v BSA, 10% v/v normal horse serum (NHS), 1% Triton X-100, in PBS containing 0.01% v/v Triton X-100 (PBS-T) for 1 hr. The sections were then blocked with Streptavidin for 15 min, followed by Biotin block for 15 min as directed by the kit manufacturer (Vector Laboratories, Burlington ON, Canada). Subsequently, the sections were incubated for 24 h at 4^{o}C in a previously characterized CYP2D6 PAb (Biomol International, Plymouth Meeting, PA, USA) diluted at 1:200 (Mann *et al.*, 2008), and glial fibrillary acidic protein (GFAP) PAb (Sigma-Aldrich Co., Mississauga ON, Canada) diluted at 1:400 in PBS-T with 1% BSA and 2% NHS. CYP2D6 was visualized using biotinylated anti-goat secondary antibody diluted 1:1000 in PBS-T (Vector Laboratories Burlington ON, Canada), peroxidase conjugated streptavidin tertiary antibody diluted 1:1000 in PBS-T (Vector Laboratories Burlington ON, Canada), and Cyanine-3 using a Tyramide-Plus signal amplification kit was used as directed by the manufacturer (PerkinElmer Life sciences, Woodbridge ON, Canada). GFAP was visualized using Alexa Fluor 488 conjugated goat anti-rabbit secondary antibody (diluted 1:1000 in PBS-T). Sections were extensively washed with PBS and then mounted onto slides for viewing. Negative control sections were incubated under the same conditions except without primary antibodies. Digital images were acquired at 2.5x,

5x, 10x, 20x, 40x and 63x magnification using a Zeiss Axio Imager Z1 microscope. Images were acquired from similar areas in each brain region simultaneously using the same duration of exposure for PD controls and PD cases.

Statistical analysis

Data were analyzed using SPSS (Chicago, Il) and Graphpad Prism Version 5 (San Diego, CA). Ontogenic expression of CYP2D6 was fitted to a fourth order polynomial curve, and the grouped age data was analyzed using one-way ANOVA. Normal distribution of samples was verified by Shapiro-Wilk and D'Agostino/Pearson omnibus K2 tests. Non-normally distributed ontogenic CYP2D6 protein expression data was normalized by square root transformation. All linear correlations were tested using Pearson's correlation followed by a two-tailed *post-test*. A linear regression model including age and genotype was used to evaluate the effect of each predictor on CYP2D6 ontogenic protein expression by use of backward selection. A two-way ANOVA was used to determine the interaction between brain regions and disease state, followed by a Bonferroni *post hoc* test. *CYP2D6 *4* allele distribution in PD cases and PD controls was analyzed using a one-tailed Fisher's test. Differences in GFAP staining between PD controls and PD cases were estimated by averaging counts of GFAP positive cells from three separate representative images acquired at 2.5x (3.6 x 2.7mm).

Results:

CYP2D6 protein expression in brain regions

Specificity for the polyclonal CYP2D6 antibody was determined by the detection of CYP2D6 in a homozygous wild-type (*CYP2D6 *1/*1*) liver and cDNA expressed CYP2D6, and the lack of detection in a homozygous null (*CYP2D6 *4/*4*) liver (**Figure 19A**). Dilution curves for two brain regions were constructed to determine the linear range of assay detection (**Figure 19B-C**). An amount of 80 μg membrane protein was determined to optimally detect CYP2D6 immunoreactive protein and any differences between ages or disease states. CYP2D6 levels varied among brain regions (**Figure 19D**), with the frontal cortex, caudate and cerebellum being significantly higher than the substantia nigra ($P<0.05$). There was no significant difference in levels of actin ($P>0.05$) between regions expressing high (frontal cortex) or low (substantia nigra) levels of CYP2D6 expression. Using immunofluorescence, CYP2D6 expression was observed in neurons as well as astrocytic cell bodies and projections (**Figure 19E**).

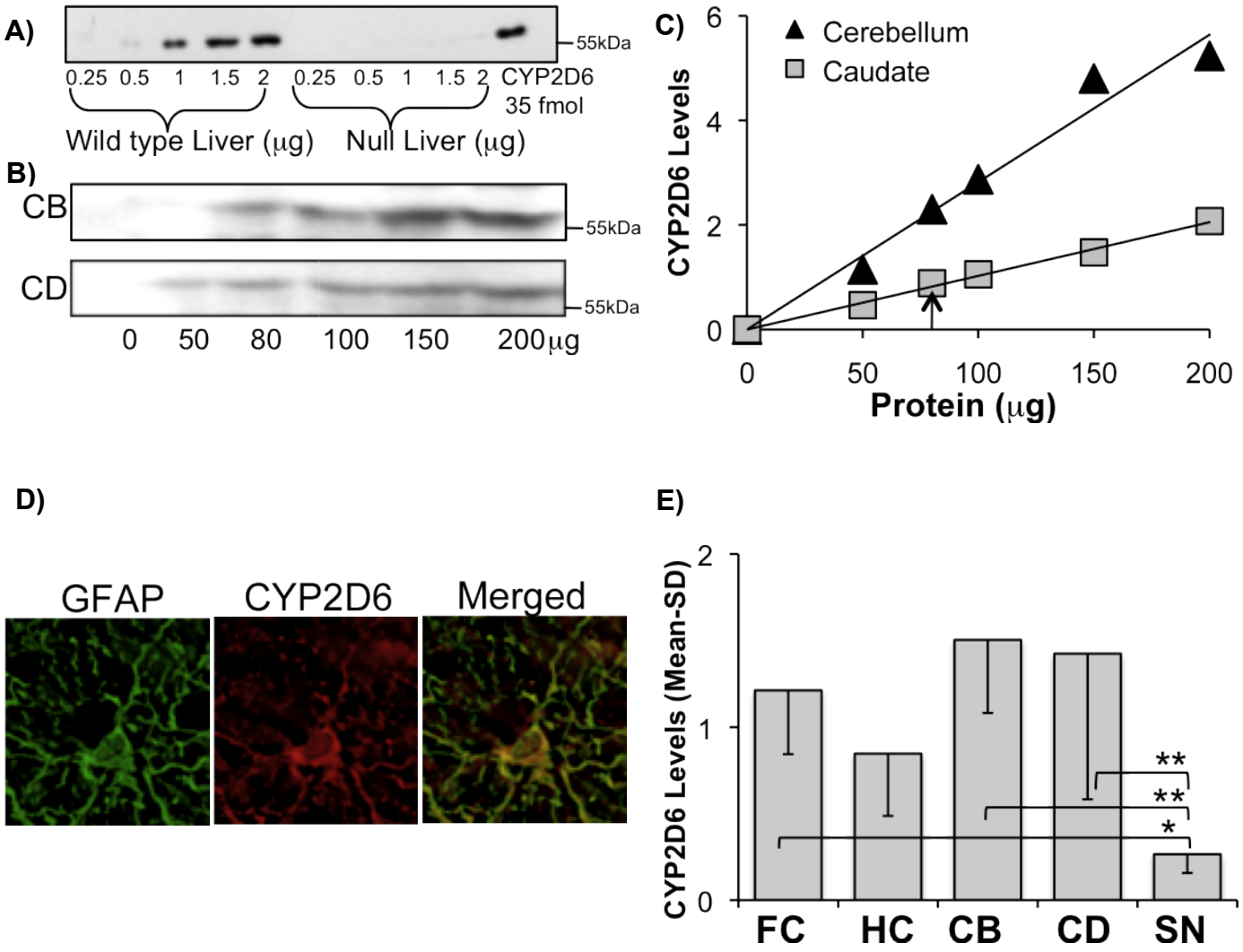

Figure 19: CYP2D6 protein detection in the brain

(A) A western blot showing CYP2D6 detection in a homozygous wild-type (*CYP2D6 *1/*1*) liver and cDNA expressed CYP2D6, with no detection of CYP2D6 in a homozygous null *(CYP2D6 *4/*4*)* liver. (**B)** Representative blots of CYP2D6 detection with increasing amounts of cerebellum (CB) or caudate (CD) membranes from control adult brain tissue. **(C)** Linear detection range for CYP2D6 in the cerebellum and caudate. The arrow indicates the amount (80 μg) of membrane protein loaded in subsequent Western blots. **(D)** Variation in the regional expression of CYP2D6 in control individuals (n=6), with a representative blot shown below. One-way ANOVA P=0.003, Bonferroni's post hoc test, F=5.19, *P<0.05, **P<0.01. FC, frontal cortex, HC hippocampus, CB cerebellum, CD caudate, and SN substantia nigra. **(E)** An astrocyte in the frontal cortex white matter showing the localization of GFAP (green) and CYP2D6 (red) in the cell body and in processes. Dashed bar= 25 μm and solid bar= 100 μM.

Ontogenic expression of CYP2D6 in the frontal cortex

The relationship between CYP2D6 protein and age was non-linear, and instead best fit the fourth order polynomial model with an R^2=0.60 (**Figure 20A inset**). As *CYP2D6* genotype can influence protein levels, we examined CYP2D6 levels in those *A)* with at least one wild-type allele (activity score 1-2), or *B)* homozygous for wild-type alleles (i.e. no altered function variants; activity score 2). Restricting analysis to only case *A* or *B* individuals, resulted in similar expression profile and increased the R^2 to 0.73 (n=58) and 0.83 (n=22) respectively. To further examine the effect of age and genotype on CYP2D6 expression, a linear regression model including *CYP2D6* genotype and age (R^2=0.34, P<0.001) showed age (β-standardized coefficient 0.59, P<0.001) significantly contributed to the observed variation in CYP2D6 expression in the frontal cortex, whereas genotype did not (β-standardized coefficient 0.03, P=0.72). There was no difference in CYP2D6 expression (P=0.70) between brain samples from CAMH and the Brain Endowment Bank when similar aged samples (24-45 yrs) were examined. Postmortem interval did not significantly affect CYP2D6 expression (P=0.82) when controlling for genotype and age in a linear regression model.

Three distinct developmental phases of cortical CYP2D6 protein expression were observed. From birth to infancy CYP2D6 expression significantly increased with age (R^2=0.68, P<0.001), while no apparent further increase (R^2=0.00, P>0.05) was observed until adulthood (**Figure 20B,C**). A second phase of increase in CYP2D6 occurred from 20 to 80 years of age (**Figure 20D**; R^2=0.42, P<0.001). Again, analyzing individuals in group *A* (activity score 1-2) or group *B* (activity score 2) as above, improved the correlation between age and CYP2D6 expression: birth to 0.9 years, R^2=0.76 (P<0.001; n=16) or R^2=0.70 (P>0.05; n=5) respectively; 20-80 years, R^2=0.43 (P=0.002, n=20) or R^2=0.72 (P>0.05; n=5) respectively.

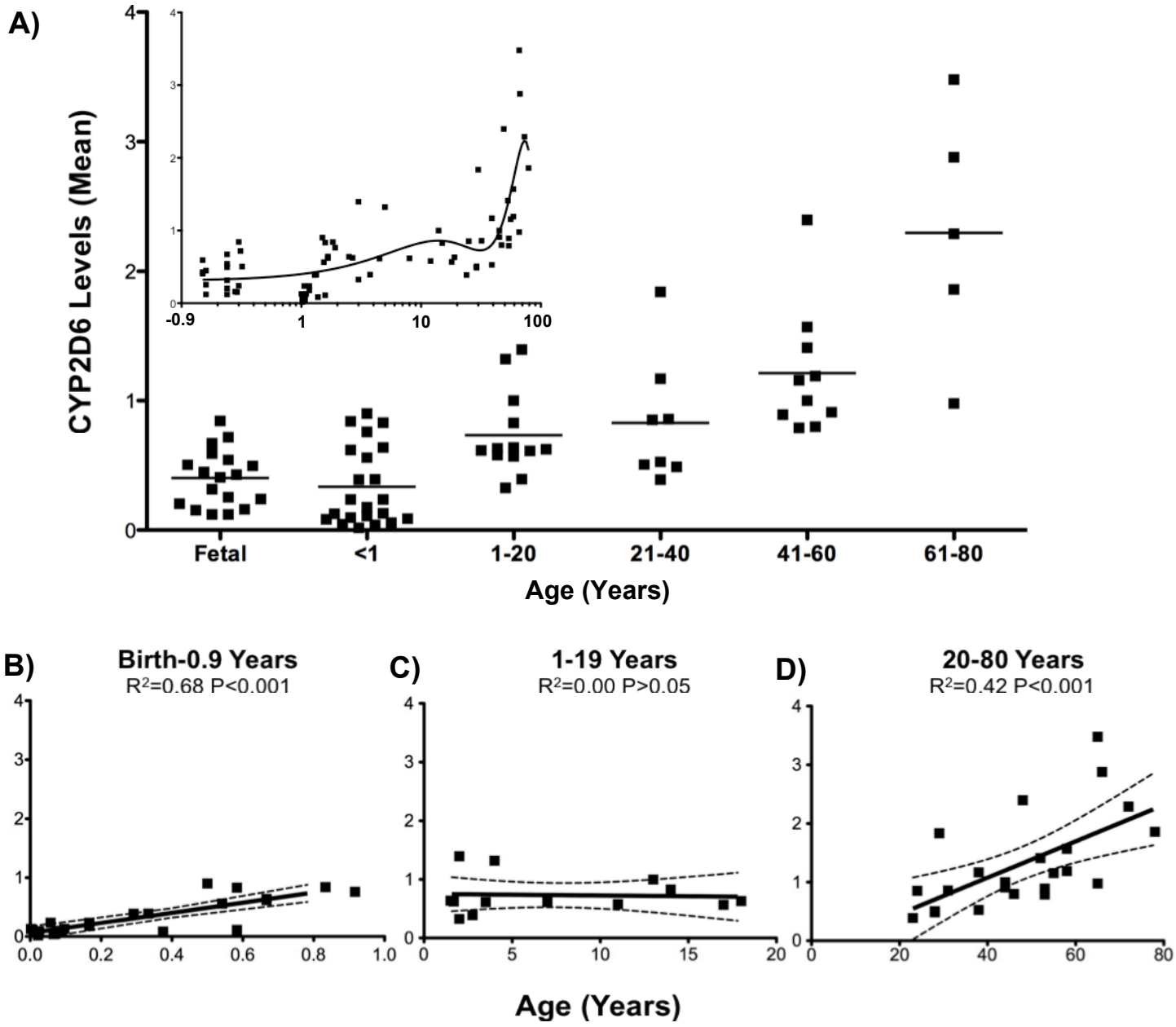

Figure 20: Ontogenic expression of CYP2D6 in the frontal cortex
(A) CYP2D6 expression varied from fetal stages (-226 to -163 d) to 78 years of age (n=76) with the line indicating the mean. One-way ANOVA $P<0.0001$ F=24.41, followed by Bonferroni's post hoc test, ***$P<0.001$ compared to fetal and <1 years of age; § $P<0.001$ compared to each prior age group. The **inset** shows the ungrouped distribution, with a fourth order polynomial curve fit. **(B-D)** Correlations of CYP2D6 protein levels and age for specific age ranges illustrating three phases of change in expression. The solid lines show the mean linear trend and the dotted lines indicate the 95% confidence intervals.

CYP2D6 levels varied among brain regions (**Figure 19C**), while levels of actin were the same between regions expressing high or low levels of CYP2D6 protein. The cerebellum had the highest and substantia nigra had the lowest levels of CYP2D6 protein in agreement with previous findings (Miksys *et al.*, 2002; Mann *et al.*, 2008). Therefore, we investigated the effect of age in three additional brain regions available. The change in adult CYP2D6 levels, of particular interest in age related diseases, was brain region specific (**Figure 21**). Similar to the frontal cortex, CYP2D6 protein increased in the substantia nigra (R^2=0.35, *P*=0.01) and cerebellum (R^2=0.37, *P*<0.01) during adulthood while in the hippocampus no increases were observed.

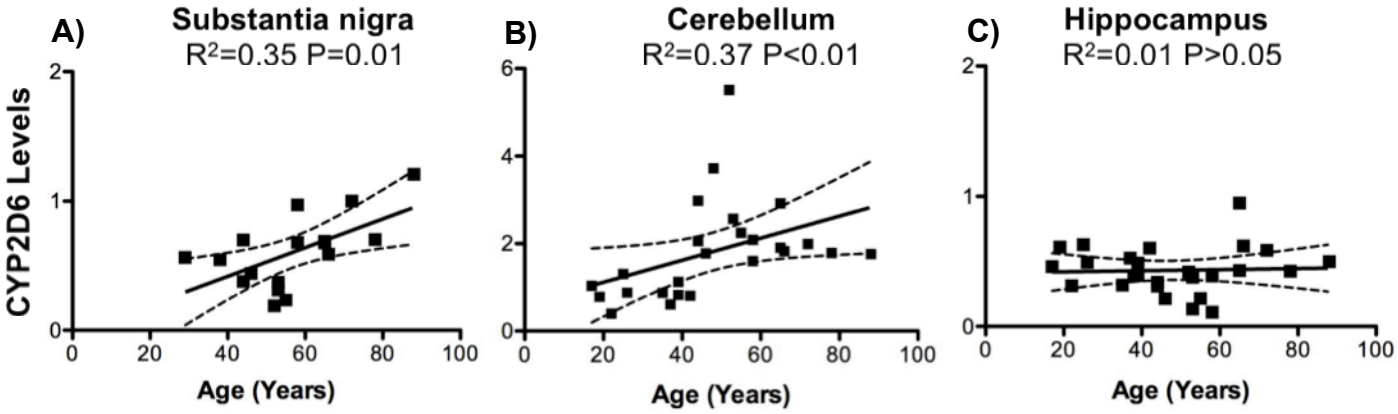

Figure 21: Influence of age on CYP2D6 expression in different brain regions
A) Substantia nigra (n=17) and **B)** cerebellum (n=25) CYP2D6 levels increase with age during adulthood, while **C)** hippocampal (n=26) CYP2D6 levels do not. The solid lines show the mean linear trend and the dotted lines indicate the 95% confidence intervals.

CYP2D6 protein expression in PD cases compared to age matched controls

As age contributed significantly to CYP2D6 protein levels, it was particularly important to use age-matched controls to compare CYP2D6 levels between PD cases and PD controls. No significant (P=0.11) difference in age was observed between PD cases (75±9 yrs) and controls (69±11 yrs) (**Table 8**). Among brain regions, compared to PD controls, PD cases had significantly lower levels of CYP2D6 (P<0.001, F=5.93, **Figure 22A**) in the frontal cortex (~51% lower, P<0.001) and cerebellum (~40% lower, P<0.001), with a similar degree of change in the hippocampus (~47% lower, P>0.05). In agreement with immunoblotting data, the frontal cortex of PD cases had lower overall CYP2D6 immunofluorescence and lower CYP2D6 cellular labeling (i.e. in pyramidal neurons) compared to PD controls (**Figure 22A inset**). Two regions directly damaged early in the etiology of PD, the caudate and substantia nigra, surprisingly showed little difference (~8% lower) between PD cases and PD controls. Of note, there was no difference in actin levels (P<0.05) between PD controls and PD cases in regions with lower (i.e. frontal cortex) or similar (i.e. substantia nigra) levels of CYP2D6.

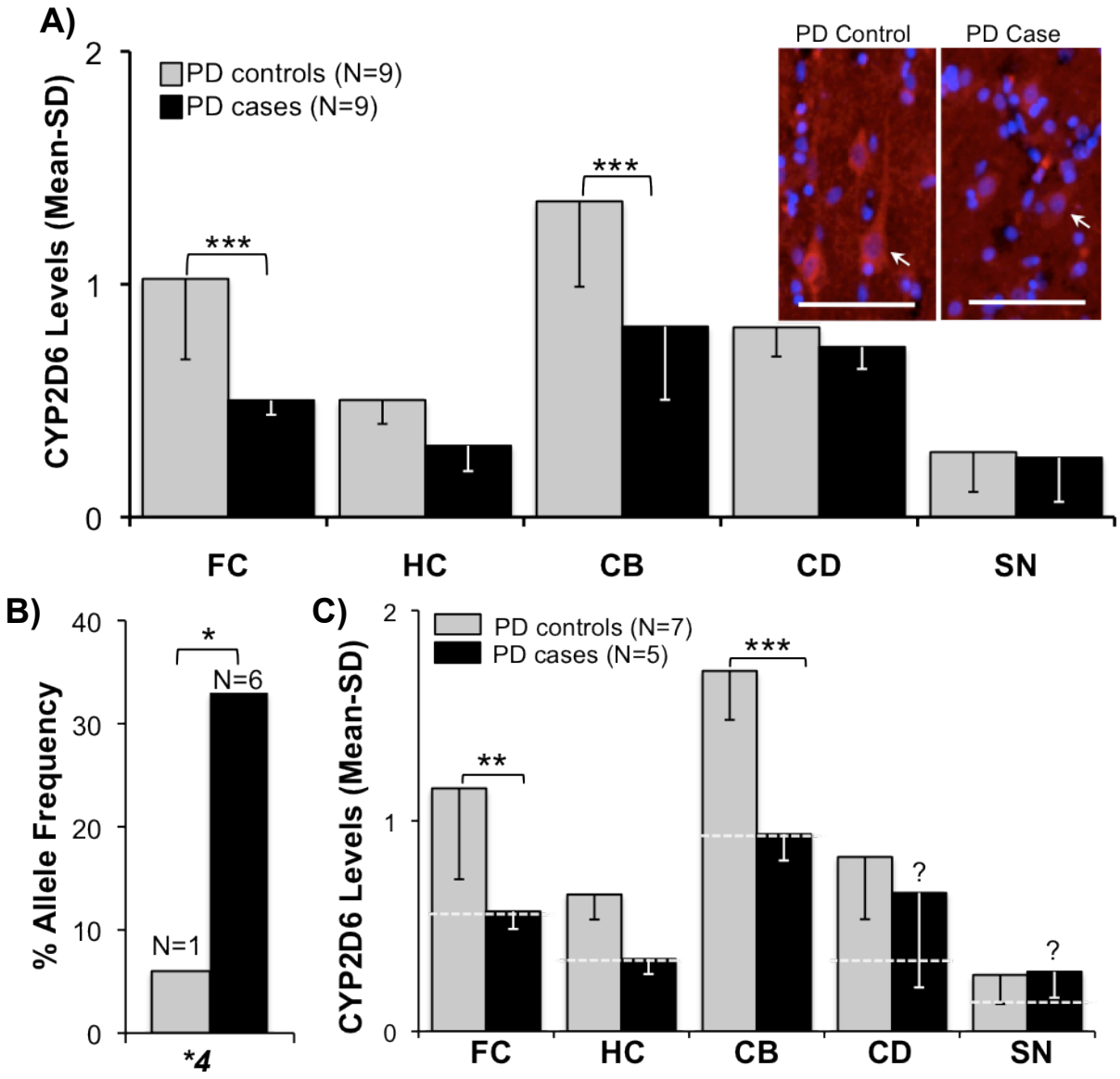

Figure 22: Lower CYP2D6 levels in PD cases and PD controls

(A) The relative levels of CYP2D6 protein in brain regions of PD cases compared to age matched controls; two-way ANOVA $P<0.001$, F=25.44. **Inset** shows greater CYP2D6 labeling (red) in a PD control sample **(left)** compared to a PD case **(right)** in frontal cortex (layers III-V) pyramidal neurons (**arrows**) and their projections. Nuclear staining indicated with DAPI (blue). **(B)** A bar graph showing the distribution of *CYP2D6 *4* null allele in controls (1 allele out of 18; gray) and PD cases (6 alleles out of 18; black), Fisher's exact test $*P=0.05$. **(C)** Among PD cases and PD controls with no null alleles, CYP2D6 levels remain lower in PD cases in the FC and CB; two-way ANOVA $P<0.001$, F=47.39. Data is expressed as the mean -S.D., Bonferroni's post hoc test $**P<0.01$, $***P<0.001$. FC, frontal cortex, HC hippocampus, CD caudate, SN substantia nigra, and CB cerebellum.

CYP2D6 genotype effect on protein expression in PD cases and PD controls

The **4* null allele, previously found to be associated with an increased risk for PD (Elbaz *et al.*, 2004; Singh *et al.*) was found at a higher frequency in PD cases compared to PD controls (Fisher's exact test P=0.05; **Figure 22B**). *CYP2D6* null genotype (activity score 0-1) distribution in this sample set was as follows: one **6/*6* and one **1/*4* individual in PD controls; one **4/*4* and three **1/*4* individuals in PD cases. As *CYP2D6* null genotype can contribute to lower levels of CYP2D6 expression, we examined protein levels in PD cases (n=5) and PD controls (n=7) that were without null alleles. Similar trends were observed of lower CYP2D6 in this subgroup as were seen in the total group: frontal cortex ~51%, P=0.05, cerebellum ~45%, P<0.001, hippocampus ~47%, P>0.05, and caudate ~22%, P>0.05 **(Figure 22C)**. These results suggest that not only may genetically reduced *CYP2D6* poor metabolizers be at increased risk for PD (Deng *et al.*, 2004; Elbaz *et al.*, 2004) but also individuals with lower CYP2D6 brain expression independent of genotype.

Gliosis and higher cellular staining of CYP2D6 in PD cases compared to controls

Since lower CYP2D6 levels are a risk factor for PD, as expected we observed ~40% lower CYP2D6 levels in cases relative to controls by immunoblotting in brain regions relatively unaffected (i.e. cerebellum) or affected in later stages during PD (i.e. frontal cortex). However we did not see lower CYP2D6 levels in regions directly affected in PD, the caudate and substantia nigra (**dotted line, Figure 22C**). To investigate further we examined PD cases versus controls for increased gliosis which occurs during PD (McGeer & McGeer, 2008; Mena & Garcia de Yebenes, 2008), This was in an attempt to explain, at least in part, higher than expected CYP2D6 levels as CYP2D6 is expressed in astrocytes (Miksys *et al.*, 2002). Indicative of gliosis, PD cases exhibited greater astrocytic GFAP staining (**Figure 23A,B**), larger astrocytes (**Figure 23A,B inset**) and more astrocytes (**Figure 23C**) compared to PD controls. CYP2D6 staining in astrocytes of PD cases (**Figure 23E**) was easily detectable and stronger than in PD controls (**Figure 23D**). In the caudate/putamen PD cases showed evidence of gliosis (**Figure 24B**) and more intense overall CYP2D6 staining in non-white matter areas. There were also noticeably greater non-astrocytic CYP2D6 cellular staining in PD cases compared to PD controls (**Figure 24A,B**). Together, PD cases had: a) a greater number of CYP2D6 positive astrocytes, b) more astrocytes in non-white matter areas, and c) more CYP2D6 positive non-astrocytic cells that were intensely stained.

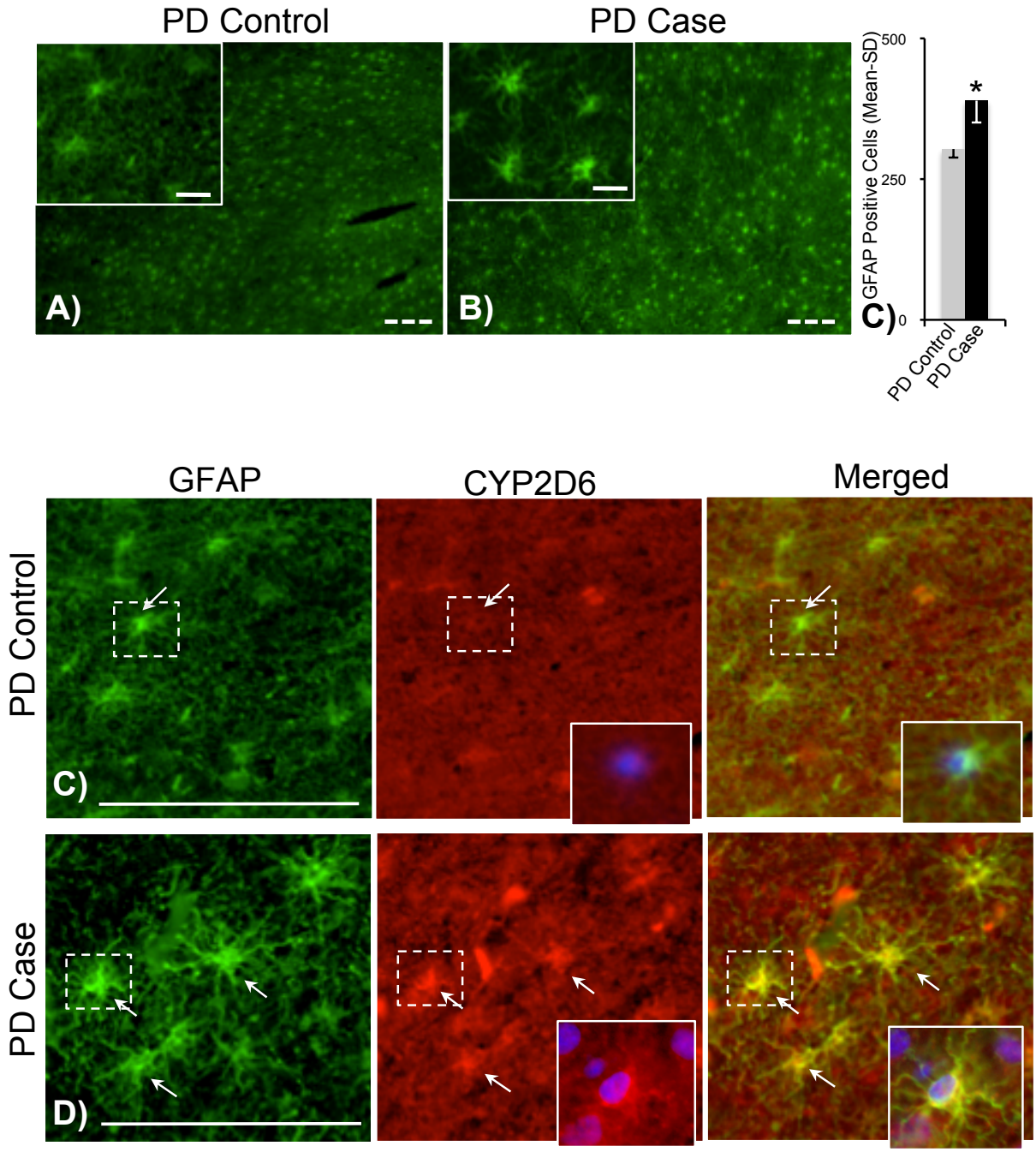

Figure 23: Gliosis and higher astrocytic CYP2D6 staining in PD cases
Compared to PD controls **(A)**, PD cases **(B)** have more astrocytes and astrocytic (**arrow**) labeling as indicated by GFAP (green). **(C)** Histogram depicting more GFAP positive cells in PD cases compared to controls. Data is expressed as the mean cell count from three separate sections – S.D., Student's *t*-Test **P*<0.05. **(D-E)** Astrocyte (GFAP in green) and cellular CYP2D6 (red) labeling was higher in PD cases **(E)** compared to PD controls **(D)**, where the merged image shows the cellular localization of CYP2D6 in astrocytes of a PD case **(E)**. No immunofluorescence was observed in the respective negative control sections. Nuclear staining indicated with DAPI (blue); solid bar= 100 μm and dashed bar =500 μm.

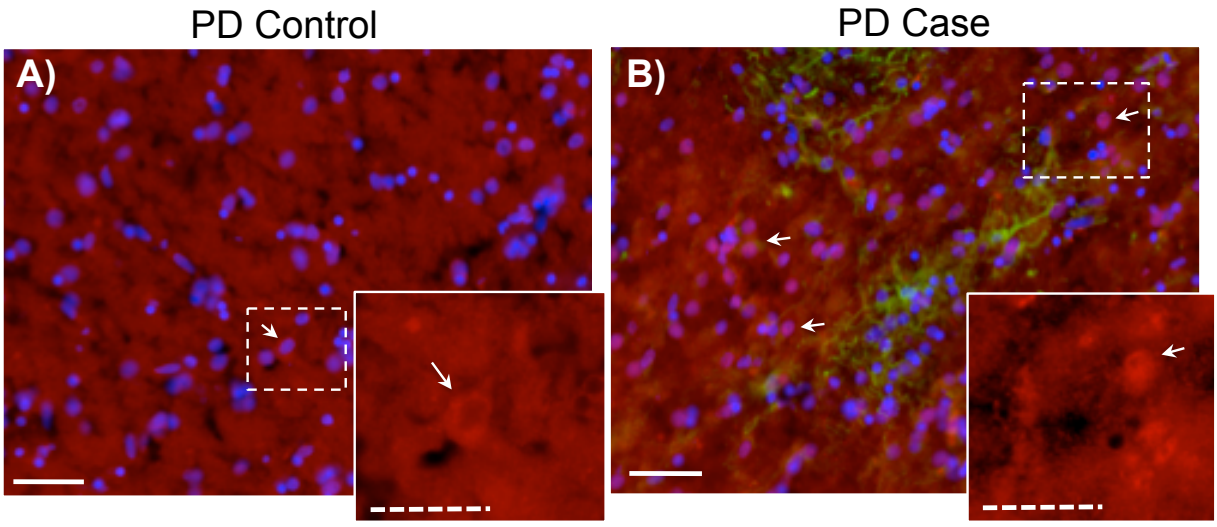

Figure 24: Neuronal and astrocytic CYP2D6 in the caudate/putamen
Compared to PD controls **(A)**, PD cases **(B)** had more intense cell specific CYP2D6 staining (**arrows**) in the caudate/putamen. Insets show cellular staining of CYP2D6 at a higher magnification within the boxed area. No immunofluorescence was observed in the respective negative control sections. Nuclear staining indicated with DAPI (blue) and GFAP staining indicated in (green); solid bar= 100 μm and dashed bar =200 μm.

Discussion:

This is the first study to measure the ontogenic expression of CYP2D6 in the human brain (fetal - 80 years), observing increased expression in the frontal cortex with age between birth and 1 year, and then again from 20 to 80 years. As CYP2D6 metabolizes a number of CNS-acting drugs (i.e. antidepressants), these changes in brain CYP2D6 levels may increase localized drug metabolism and consequently alter drug effect with increasing age. This study is also the first to show PD cases have ~40% lower levels of brain CYP2D6 compared to controls; even when controlling for *CYP2D6* genotype. The lower levels in the cerebellum, a region not effected in PD, suggests that low CYP2D6 expression likely precedes the disease rather than being a result of PD. Seeing that CYP2D6 inactivates a number of neurotoxins would suggest individuals with low CYP2D6 levels may be more sensitive to PD-causing neurotoxins predisposing them to PD, compared to those who have high levels of brain CYP2D6.

High levels of CYP2D6 have been reported in smokers, particularly in the basal ganglia, consistent with smokers having a reduced risk for developing PD (Alves *et al.*, 2004; Mann *et al.*, 2008). Nicotine, a neuroprotective agent in cigarette smoke, significantly induces CYP2D6 in the basal ganglia, especially in the substantia nigra, in non-human primates (Mann *et al.*, 2008). In addition, over-expression of CYP2D6 protects PC12 cells from MPP^+ toxicity (Matoh *et al.*, 2003) whereas inhibiting CYP2D6 activity in human neuroblastoma cells increases neurotoxicity by MPP^+ (Mann *et al.*, submitted). Thus, the induction of brain CYP2D6 may be one mechanism by which smoking and nicotine protect against PD. These observations taken together with the knowledge that *CYP2D6* genetically poor metabolizers are at increased risk for PD, suggest that the lower CYP2D6 expression in PD cases is likely contributing to the risk for PD rather than being a result of PD.

In contrast to lower CYP2D6 in regions affected minimally or in later stages of PD (i.e. cerebellum and frontal cortex), the substantia nigra and caudate did not show significantly lower CYP2D6 levels in PD cases. Previous studies indicate that in both human PD cases and in PD animal models, regions of damage undergo gliosis (McGeer & McGeer, 2008). Gliosis involves glial cell growth, replication and migration to damaged areas of the brain (McGeer & McGeer, 2008; Mena & Garcia de Yebenes, 2008). In agreement with these findings we noted more and larger astrocytes in PD cases compared to PD controls. Additionally, we noted more strongly labeled CYP2D6 positive astrocytes plus intense staining of other cell types in PD cases. Therefore in response to gliosis or neuronal damage CYP2D6 may be induced in astrocytes or neurons, which may have minimized the difference observed in CYP2D6 levels in the caudate and substantia nigra between PD cases and PD controls.

During PD, the substantia nigra is known to exhibit a progressive loss, of between ~6-13% per year, of dopaminergic neurons in contrast to aging alone where the loss is less than 1% per year (Marek *et al.*, 2003; Hindle, 2010). This region in particular is known to be extremely vulnerable to toxins and interestingly has relatively low levels of CYP2D6 expression compared to other brain regions (Miksys *et al.*, 2002; Hindle, 2010). These lower CYP2D6 levels may reduce the ability of the substantia nigra to detoxify neurotoxins putting this region at greater risk of damage. CYP2D6 in this region is also highly responsive (i.e. inducible) to factors such as alcohol, smoking and nicotine (Mann *et al.*, 2008). Collectively, the observed increase of CYP2D6 expression in the substantia nigra during adulthood may be a protective response to accumulating toxins, a compensatory response to signaling from age-related neurodegeneration processes, and/or a response to xenobiotics/drugs.

Xenobiotics can up- or down-regulate CYPs in an organ- and brain region-specific manner (Miksys & Tyndale, 2004). For example, CYP2D6 is not inducible in the liver but can

be induced in select brain regions by nicotine (Mann *et al.*, 2008). Factors such as genetic variation, age, brain region, and smoking can all influence CYP2D6 brain levels. When the effect of age and genotype was assessed in the frontal cortex, age significantly influenced CYP2D6 expression contributing substantially to the variation in CYP2D6 expression in this region. The increase in CYP2D6 with age, which was also observed in other regions from 20-80 years of age, may be a natural phenomena of aging like the changes seen in brain volume, white matter, myelinated fibers, and the overall loss of neurons (~10%) (Piguet *et al.*, 2009). However, the differential effects of both age and disease on CYP2D6 expression between brain regions suggests that CYP2D6 may be regulated specifically and/or varies in sensitivity to environmental factors by brain region (Miksys *et al.*, 2002; Miksys *et al.*, 2005; Mann *et al.*, 2008). While it is unclear what causes the increase of CYP2D6 with age, these changes in brain CYP2D6 may result in altered localized metabolism of CYP2D6 substrates.

CYP2D6 metabolizes ~30% of prescribed drugs, a number of which act on the CNS (Zanger *et al.*, 2004). As a result, the increase in brain CYP2D6 in adulthood may also influence localized drug metabolism and drug effect. Recently we have shown in a rat model that altering CNS, but not hepatic, CYP2B drug metabolism altered the pharmacological response to propofol; suggesting that altered levels of CYP in the brain can consequently influence drug effect (Kokhar & Tyndale, 2010). Older patients (>75 years) on the antidepressant drug desipramine, a CYP2D6 substrate, were less responsive than younger patients, when controlling for drug dose and plasma levels (Nelson *et al.*, 1995). As 60-80 year old individuals have high levels of brain CYP2D6, higher rates of localized CNS drug metabolism may contribute to the reduced response. Overall, the higher levels of brain CYP2D6 between 40-80 years of age may render this age group more or less responsive to

different CNS drugs, depending on their activation (i.e. codeine) or inactivation (i.e. anti-depressants) by CYP2D6.

In addition to CNS drugs CYP2D6 can also metabolize neurotransmitters including serotonin (Yu *et al.*, 2003b) and dopamine (Thompson *et al.*, 2000), therefore changes in brain CYP2D6 expression may affect brain function. In fact, an imaging study showed an effect of *CYP2D6* genotype on resting brain perfusion (Kirchheiner *et al.*, 2010). Moreover, *CYP2D6* genotype has been associated with personality traits, where poor metabolizers are likely to have anxious and less sociable personalities (Gonzalez *et al.*, 2008). Altogether supporting a role for CYP2D6 in the metabolism of neurotransmitters by CYP2D6 in the brain. Of relevance to PD, dopamine auto-oxidation and metabolism can generate reactive oxygen species and dopamine derivatives that are neurotoxic (Graham, 1978; Asanuma *et al.*, 2004; Greggio *et al.*, 2005). CYP2D6's ability to metabolize dopamine and other endogenous neurotoxins (i.e. β-carbolines) could possibly alleviate some of this toxic burden, especially in individuals with high brain CYP2D6 expression.

In conclusion, human brain CYP2D6 expression increased with age, and CYP2D6 expression was lower in PD patients compared to controls in areas affected minimally until late stages of PD. In contrast, there was no difference in CYP2D6 levels in the caudate and substantia nigra of PD cases. This is likely due to CYP2D6 induction in remaining neuronal cells and astrocytes as well as expression in other glial cells that increase in a diseased state. Genetic association studies indicate an increased risk for PD in people who have genetically lower CYP2D6 activity. This study provides the first evidence that PD cases express lower brain CYP2D6 levels, which may compromise their ability to inactivate PD-causing neurotoxins. Together, these findings suggest that brain CYP2D6 could play an important role

in neuroprotection and that interindividual variation in the expression of this enzyme may alter risk for PD.

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Significance of chapter

This is the first study to measure the ontogenic expression of CYP2D6 in the human brain (fetal to 80 years). In contrast to liver, CYP2D6 in the brain increased with age during adulthood and the effect of age was brain region specific. As CYP2D6 metabolizes a number of CNS-acting drugs (i.e. antidepressants) select age groups may respond differently to the drugs as a result of age-related differences in brain CYP2D6 levels altering localized drug metabolism. These results also indicate the importance of using age-matched controls when assessing brain CYP2D6 levels.

Most studies indicate that *CYP2D6* genetically poor metabolizers are at higher risk for developing PD. This study is the first to show that PD patients *also* express lower (~40%) levels of brain CYP2D6 compared to age-matched controls. Analyzing only *CYP2D6* wild-type individuals did not change our findings, suggesting that not only are PMs at higher risk for PD, but also EMs with lower expression of brain CYP2D6. Since CYP2D6 inactivates a number of neurotoxins, individuals with low CYP2D6 levels might be more sensitive to PD-causing neurotoxins predisposing them to PD compared to those who have high levels of brain CYP2D6. These results present another line of evidence that CYP2D6 in the brain may be involved in neuroprotection against PD.

Section 3: General Discussion

1. Study models

Epidemiological and post-mortem studies are important for identifying factors associated with variation in health or disease (e.g., CYP2D6 with PD). Given genetic variability and possible exposures to environmental factors, *in vitro* and *in vivo* models are often needed for confirmation of identified risk factors and clarification of mechanisms (Wehling, 2009; Wendler & Wehling, 2010). While *in vitro* models can be used to study the impact of drugs at the cellular and molecular level, *in vivo* models are necessary to predict drug outcomes within the whole, and more complex, organism (Hewitt *et al.*, 2007; Capitanio & Emborg, 2008; Wendler & Wehling, 2010; Xie *et al.*, 2011). In our studies we utilized both an *in vitro* and an *in vivo* model to characterize some potential aspects of the relationship between brain CYP2D6 and PD.

1.1 SH-SY5Y human neuroblastoma cells as a model

The SH-SY5Y human neuroblastoma cell line is commonly used to model PD in culture because it has the characteristics of dopaminergic neurons. SH-SY5Y cells express DA receptors and key dopaminergic enzymes, including tyrosine hydroxylase, DA decarboxylase, and DA β-hydroxylase (Riveles *et al.*, 2008; Xie *et al.*, 2011). These cells also express the DAT, which is essential for MPP^+ toxicity (Notter *et al.*, 1988; Xie *et al.*, 2011). Similar to dopaminergic neurons in the brain, SH-SY5Y cells are sensitive to commonly used PD toxins, such as rotenone, paraquat, MPP^+, and 6-OHDA (Klintworth *et al.*, 2007; Riveles *et al.*, 2008; Watabe & Nakaki, 2008; Mann & Tyndale, 2010).

Other advantages of using SH-SY5Y cells is that this cell line expresses endogenous CYP2D6. In **Chapter 1** we demonstrated that these cells could metabolize the CYP2D6-

specific substrate AMMC (Mann & Tyndale, 2010). Further, SH-SY5Y cells in culture were previously shown to metabolize codeine to morphine, a reaction primarily conducted by CYP2D6 (Poeaknapo *et al.*, 2004; Boettcher *et al.*, 2005). Collectively, these results suggest that SH-SY5Y neuronal cells express functional CYP2D6 and the co-enzymes (e.g., CYP reductase) required for CYP2D6 function.

Disadvantages to using this cell line as a PD model. *In vitro* models may not always reflect the intact brain and the variable composition of MPTP activating (i.e., MAO) and inactivating enzymes (i.e., CYP2D6). Using this cell line, we found relatively low levels of CYP2D6 expression in comparison to CYP3A. This is in contrast to the brain where there is greater expression of CYP2D6 when compared to other common hepatic drug metabolizing enzymes such as CYP3A (Dutheil *et al.*, 2009; Dutheil *et al.*, 2010). The discordance between brain and SH-SY5Y cell CYP expression may be because the cell line is ganglionic in origin (Xie *et al.*, 2011). Therefore, the CYP2D6 and CYP3A expression in SH-SY5Y cells may represent the relative CYP expression observed in sympathetic and/or parasympathetic ganglions. Other disadvantages of using this cell line for a PD model include the likelihood of unknown cell heterogeneity in culture over time and the possibility that assessing cell death in a constantly replicating cell environment may be different from results in non-replicating cells, such as primary neurons (Xie *et al.*, 2011).

Regardless of the disadvantages, this cell line provides a good model of dopaminergic neurons that may be useful for assessing the cellular expression and impact of CYP enzymes. The expression of CYP reductase, a co-enzyme required for CYP activity, has been observed to varying degrees among different brain regions (Norris *et al.*, 1994; Dutheil *et al.*, 2008). Future studies could utilize SH-SY5Y cells, or primary neuronal cells, to study CYP and CYP reductase cellular organelle expression, localization, and interactions using techniques such as

fluorescent-tagged antibodies or transfecting fluorescent-tagged CYP proteins. Transfecting with fluorescent-tagged proteins would allow for the tracing of CYP enzyme trafficking and targeting to different organelles and possibly for determining the interaction between CYP reductase and CYPs in neurons using live fluorescence microscopy, and/or FRET. Neuronal cell cultures may also be useful in determining the mechanisms of CYP2D6 regulation in neuronal cells. SH-SY5Y cells express α3, α5, α7, β2 and β4 subunits of the nAChR. As nicotine can induce brain CYP2D6 (**Chapter 2**), using SH-SY5Y cells we can determine if this induction is a nAChR dependant or independent pathway (Ridley *et al.*, 2001; Serres & Carney, 2006; Xie *et al.*, 2011).

1.2 Non-human primates as *in vivo* models

Results obtained from *in vitro* studies do not always mirror what may be occurring *in vivo*, thus an *in vivo* model is essential to assess outcomes in a whole organism (Hewitt *et al.*, 2007). The use of monkeys as *in vivo* models is ideal because compared to other species (e.g., rats) they are most similar to humans in terms of behaviour, physiology, drug metabolism, neuroanatomy, and neurotoxin response (Capitanio & Emborg, 2008; Wendler & Wehling, 2010). Although this model is costly, has ethical constraints, and monkeys are difficult to handle compared to rodents, the benefits out weigh the disadvantages and it is often used to model human behaviour and disease.

Like humans, monkeys present age-related dysfunction of the nigrostriatal system associated with motor impairments such as tremors and balance disturbances (Capitanio & Emborg, 2008; Hurley *et al.*, 2011). Old monkeys, including old AGMs, form β-amyloid plaques (associated with gliosis and neuritic dystrophy) making them good models to study neurodegenerative disease (e.g., Alzheimer's disease), prevention, and it's treatment (Lemere *et al.*, 2004). Further, neurotoxin treatments in monkeys precipitate similar clinical symptoms

to those observed in PD; monkeys show higher sensitivity than rodents to toxins such as MPTP, and monkeys exhibit dyskinetic side-effects of the PD treatment *L*-Dopa, as observed in PD cases (Langston *et al.*, 1984d; Yoshida *et al.*, 1993; Quik *et al.*, 2007b). Overall, monkeys are a valuable tool to assess potential neuroprotective agents to halt or slow the progression of PD and drug treatments that can provide symptomatic relief.

An additional advantage of using monkeys is that their CYPs have high sequence homology (>90%) to human CYPs (Hichiya *et al.*, 2004; Iwasaki & Uno, 2009). They metabolize probe substrates using similar metabolic pathways and display similar chemical inhibition profiles. Like human CYP2D6, monkey CYP2D mostly *O*-demethylates dextromethorphan, *1'*-hydroxylates bufuralol, and *4*-hydroxylates propranolol (Narimatsu *et al.*, 2010; Uno *et al.*, 2010). Notably, monkey CYP2D metabolism is inhibited by the potent CYP2D6 inhibitor quinidine, whereas rat CYP2Ds are potently inhibited by quinine, a stereoisomer of quinidine (Ai *et al.*, 2009; Shimizudani *et al.*, 2010). Another advantage of using monkeys as *in vivo* models is that they metabolize nicotine primarily by CYP2A as opposed to rats which use a CYP2B enzyme (Hammond *et al.*, 1991; Schoedel *et al.*, 2003). This may be important if specific metabolites of nicotine are also responsible for brain CYP2D induction in primates.

We previously investigated the expression of brain CYP2D in both rat and human post-mortem tissue (Miksys *et al.*, 2000b; Miksys *et al.*, 2002; Yue *et al.*, 2008). Of the regions assessed in both, rat brain CYP2D had a different expression profile when compared to human brain CYP2D6. Brain regions demonstrating an induction of CYP2D in rats upon chronic nicotine treatment were also different from those associated with smoking in humans (i.e., the striatum and hippocampus) (**Figure 10**) (Mann *et al.*, 2008; Yue *et al.*, 2008). Therefore, to better understand human brain CYP2D6, a monkey model was used to investigate the primate

brain CYP2D expression and nicotine induction patterns in a controlled environment, and to determine if nicotine is responsible, at least in part, for the higher brain CYP2D6 levels observed in smokers (Mann *et al.*, 2008).

2. Variation in CYP2D6 expression

Hepatic CYP2D6 displays a large amount of interindividual variability in protein expression and activity in humans (Zanger *et al.*, 2001; Gaedigk *et al.*, 2008). The interindividual differences in CYP2D6 are thought to be largely due to genetic variation. This is because hepatic CYP2D6 is relatively uninducible by classic CYP inducers (e.g., phenobarbital), and the *CYP2D6* gene is highly polymorphic (Coutts & Urichuk, 1999; Zanger *et al.*, 2004; Gaedigk *et al.*, 2008). Other than *CYP2D6* genetics, some sources of variation that could alter hepatic CYP2D6 expression include interindividual differences in expression of transcription factors that regulate CYP2D6 and/or environmental factors.

Hepatic CYP2D6 expression is regulated by HNF-4α, and this transcription factor expression is shown to correlate with CYP2D6 expression (Cairns *et al.*, 1996; Corchero *et al.*, 2001). For example, the highest level of CYP2D6 expression is observed in the liver and kidneys, which also express the highest levels of HNF-4α (Gonzalez, 1990; Xie *et al.*, 2009). Moreover, individuals with genetic variants in *HNF-4α*, resulting in a protein that is unable to bind to the recognition site in the CYP2D6 promoter, have reduced CYP2D6 activity compared to those with wild-type *HNF-4α* (Lee *et al.*, 2008b). HNF-4α expression in HepG2 cells was affected by select microRNAs which could also contribute to the variation in HNF-4α expression and therefore variation in hepatic CYP2D6 expression (Ramamoorthy A. *et al.*, 2008). A similar effect has been observed for CYP3A4, where transcription was influenced by microRNA dependent changes in the transcription factor Pregnane X receptor, a known regulator of CYP3A4 (Ingelman-Sundberg *et al.*, 2007; Takagi *et al.*, 2008; Pan *et al.*, 2009).

Altogether the evidence suggests that CYP2D6 expression could change as a result of variability in the expression and function of HNF-4α.

Some evidence also suggests that environment can influence CYP2D6 activity. For example, individuals of the same genotype from different parts of the world show varying degrees of CYP2D6 substrate metabolism (Ingelman-Sundberg, 2005). One study showed native Ethiopians were faster metabolizers for CYP2D6 than native Ethiopians living in Sweden with the same *CYP2D6* genotype; and native Ethiopian residents of Sweden were faster metabolizers than native Swedes with the same *CYP2D6* genotype (Aklillu *et al.*, 2002). As CYP2D6 has a high affinity for plant alkaloids, local diet and non-dietary exposures may influence CYP2D6 activity (Cascorbi, 2003; Ingelman-Sundberg, 2005).

In **Chapter 2** and **Chapter 3** we report that, like hepatic CYP2D6 levels, human brain CYP2D6 expression varies greatly between people. However, unlike hepatic CYP2D6, human brain CYP2D6 changes with age and altered brain CYP2D6 expression is associated with environmental factors such as smoking (Mann *et al.*, 2008; Mann *et al.*, submitted). Variation in human brain CYP2D6 expression is therefore likely due to a combination of genetic variation and environmental factors (i.e., inducers and/or inhibitors).

In **Chapter 2** we demonstrated that brain CYP2D expression varies between monkeys, similar to that observed between humans (Mann *et al.*, 2008). Like human CYP2D6, sources of this inter-animal variation in brain CYP2D expression could be a result of genetic variation. Genetic polymorphisms have been reported in AGM, macaques, and cynomolgus monkeys. Gene copy variants for *CYP2D* have been observed in cynomolgus and marmoset monkeys (Uno *et al.*, 2010; Uno *et al.*, 2011). AGMs have genetic polymorphisms that contribute to variation between animals in the expression of genes implicated in apoptosis and immune response (Jasinska *et al.*, 2009). Polymorphisms have been found in the DA D_4 receptor of

AGMs (Bailey *et al.*, 2007). Further, there are noted differences in DA metabolism between AGMs, predicted to be a result of genetic variants (Fairbanks *et al.*, 1999). Collectively this suggests that, like humans, genetic variation occurs in monkeys, which may contribute to the observed differences in brain CYP2D expression and/or sensitivity to inducers between animals. If CYP2D in AGMs is indeed as genetically variable as in humans, future studies should identify monkeys with *CYP2D* null variants to assess differences in vulnerability to MPTP toxicity compared to monkeys with wild-type *CYP2D* and those with gene copy number variants of *CYP2D*.

2.1 Factors contributing to regional variation

In **Chapter 3**, we found that human brain expression of CYP2D6 is highest in the cerebellum and lowest in the SN (Mann *et al.*, submitted). This is similar to what we observed previously (Miksys *et al.*, 2002). In saline-treated monkeys, the basal expression was highest in the brainstem and lowest in the hippocampus (Mann *et al.*, 2008). There was good overlap in CYP2D expression across different brain regions between non-smoking humans and saline-treated monkeys; however, in comparison, human hippocampus had moderate levels of CYP2D6 and the levels in the brainstem were undetermined. CYP2D6 levels in the SN were the lowest in non-smoking humans but second highest in saline-treated monkeys. This lower expression in the SN may be specific to humans because rats, like monkeys, also show high levels of CYP2D in the SN (Bromek *et al.*, 2010). CYP2D activity in the rat SN was reported to be similar to the cerebellum, a region that expresses the highest CYP2D protein and activity levels in the rat brain (Tyndale *et al.*, 1999; Yue *et al.*, 2008; Bromek *et al.*, 2010).

In monkeys, the regional variation in brain CYP2D expression and in the induction profile may be influenced by the expression of alternative CYP2D isoforms and/or isoenzymes in the brain. Using western blotting, in **Chapter 2,** we detected a single CYP2D band in the

monkey liver and three immunoreactive CYP2D bands (molecular weights of 51, 52, and 55 kDa) in the brain. These bands were detected using both unpurified and monkey liver purified polyclonal antibodies, suggesting the possibility of alternative forms of the same CYP2D in the brain. The lower band (51 kDa) may be an mRNA splice variant product, and the upper band (55 kDa) may be a post-translationally modified CYP2D or a brain-specific isoenzyme of CYP2D in the monkey brain. Two distinct isoenzymes of CYP2D have been identified in both cynomolgus and marmoset monkeys, and a brain-specific CYP2D isoenzyme (CYP2D18) has been described in rats (Kawashima *et al.*, 1996; Hichiya *et al.*, 2004; Uno *et al.*, 2010). Notably, upon chronic nicotine treatment in our AGMs, induction was observed in the middle band (52 kDa), but there was no change detected in the upper band (55kDa). This suggests the two CYP2D proteins represented by the upper and middle bands are regulated differently by nicotine in the monkey brain. Induction of select isoenzymes in the brain upon drug treatment has been previously reported in rats, which express multiple isoforms of CYP2D in the brain. In male Wistar rats, for example, castration induced brain CYP2D5 and chronic treatment with clozapine induced CYP2D4 (Riedl *et al.*, 1999).

In addition to region-specific expression, CYP2D6 is expressed in select cell types. We and other groups have shown that CYP2D6 is expressed in both neuronal and non-neuronal cells, including pyramidal neurons, granular cells, Purkinje cells, glial cells, and astrocytes (Gilham *et al.*, 1997; Siegle *et al.*, 2001; Miksys *et al.*, 2002). The contrasting expression observed across different brain regions may be influenced by the different, and select, population of cells that occupy specific brain regions. Pyramidal neurons, for example, are found mostly in the cortex, hippocampus, and amygdala, and Purkinje cells are found in the cerebellum (Miksys *et al.*, 2002; Spruston, 2009). Further, some neurons project within a specific region (e.g., pyramidal neurons project within the cortex) while others project from

one region to another (e.g., dopaminergic neurons in the SN project to the striatum). Notably, CYP2D6 is expressed both in neuronal cell bodies and in their projections (Miksys *et al.*, 2002; Mann *et al.*, 2008). If CYP2D6 is differentially expressed within a cell (i.e., cell body versus axon terminal), then the level of CYP2D6 expression could be altered by which brain region the projections begin or end in. Thus, to better characterize cellular expression of brain CYP2D6, future studies should co-label for specific neuronal cell markers and CYP2D6.

3. Factors contributing to altered brain CYP2D6 expression

3.1 Environmental influences on brain CYP2D6

Unlike hepatic CYP2D6, brain CYP2D6 is inducible. In humans, alcohol and smoking are associated with higher brain CYP2D6 levels, especially in select regions of the basal ganglia and cerebellum (Miksys *et al.*, 2002; Miksys & Tyndale, 2004; Mann *et al.*, 2008). In **Chapter 2**, we show that monkey brain CYP2D is induced in a region-specific manner by nicotine, a component of cigarette smoke. Further, CNS-acting drugs, such as clozapine, fluoxetine, thioridazine, and nicotine, can induce CYP2D in a region-specific manner in the rat brain (Mizuno *et al.*, 2003a; Yue *et al.*, 2008; Haduch *et al.*, 2010).

The inducer nicotine is of particular interest as many people are exposed to nicotine to varying degrees through the use of cigarettes or through second-hand smoke (passive smoking) (Henningfield *et al.*, 1985). Nicotine in the form of a clinical therapeutic is commonly used as a smoking cessation drug (Grief, 2011). It has also been tested in clinical trials as a therapeutic for ulcerative colitis, Alzheimer's disease, PD, and age-associated cognitive impairment (White & Levin, 1999; Kelton *et al.*, 2000; Min *et al.*, 2001; Guslandi *et al.*, 2002; White & Levin, 2004).

We found significantly higher levels of CYP2D6 in the SN, globus pallidus, and cerebellum in human smokers (Mann *et al.*, 2008). We also demonstrated that nicotine can induce monkey brain CYP2D in regions similar to those in which higher levels were observed in human smokers. Specifically, nicotine induced CYP2D in the structures of the basal ganglia (caudate and putamen), including the SN, in the monkey brain. Although the monkey cerebellum did not show CYP2D induction by nicotine using western blotting, we did observe cellular induction using immunohistochemistry. Notably, the monkey brain showed significant reduction in CYP2D upon nicotine treatment in select brain regions (i.e., the thalamus and parietal cortex). Brain region-specific reduction in CYP2D protein has also been observed with clozapine treatment in rats (Haduch *et al.*, 2010). In this study with rats, CYP2D was induced in the cerebellum, but CYP2D protein was reduced in the nucleus accumbens and striatum. Together these results suggest that CYP2D protein in the brain is under complex regulation that is both brain region- and drug-specific.

In human smokers, only the SN and the globus pallidus structures of the basal ganglia showed higher levels of CYP2D6 when compared to non-smokers. The failure to observe a significant influence of smoking on CYP2D6 in the caudate and putamen, in contrast to what was observed in nicotine-treated monkeys, might be due to the small sample size in this study (non-smoking non-alcoholics = 3 and non-alcoholic smokers = 10). Another reason for the differences in CYP2D expression between human smokers and nicotine-treated monkeys may be because monkeys were kept in a controlled environment. This is in contrast to humans that are exposed to uncontrolled variables, such as diet, use of clinical drugs, and lifestyle factors. Further, monkeys were not exposed to cigarette smoke, which suggests that there may be additional compounds in the smoke from cigarettes that may regulate CYP2D in the brain. The discrepancy in regions where nicotine induced brain CYP2D could also be due to species

differences (humans *versus* monkeys) although this should be minimal, or differences in duration of drug exposure (years *versus* days). Additionally, as age can affect brain CYP2D6 levels, the difference in the age of monkeys (early adulthood) and humans (early to late adulthood) may contribute to the contrasting expression and/or induction of brain CYP2D6 expression.

The brain region-specific changes in CYP2D expression induced by smoking or nicotine may be a result of select nAChR subtype activation. There are multiple nAChR subtypes that show diverse expression across different brain regions (Gotti & Clementi, 2004; Quik *et al.*, 2007a). Nicotine treatment in monkeys induced CYP2D specifically in brain structures of the basal ganglia, which has the highest expression of α3/α6* nAChR subtype (Gotti *et al.*, 2006; McCallum *et al.*, 2006a). Therefore, α3/α6* nAChR subtype *may* be involved in the regulation of CYP2D6 and should be considered in future studies.

The mechanisms of brain CYP2D induction by smoking or nicotine are currently unknown. In rats chronically treated with nicotine, no changes in brain CYP2D mRNA levels are observed, suggesting the mechanism of induction is likely due to post-transcriptional modifications (Yue *et al.*, 2008). CYP2D protein levels peak at 8 hours post 7 day nicotine treatment and return back to basal levels by 12 hours in all three regions of induction (cerebellum, hippocampus, and striatum) studied (Yue *et al.*, 2008). In contrast to brain CYP2D, rat brain CYP2B can be transcriptionally induced by nicotine but displays a different pattern of protein induction time course than CYP2D (Miksys *et al.*, 2000a; Khokhar *et al.*, 2010). Post nicotine–treatment in rats, brain CYP2B protein peaks at an earlier time point and returns to baseline at 7 days (Khokhar *et al.*, 2010). The contrasting mechanism of induction and induction profile between CYP2B and CYP2D implicate that nicotine likely regulates brain CYP2D through post-transcriptional mechanisms.

Post-transcriptional changes that could result in the induction of brain CYP2D include decreased splicing, increased translation, the enhancement of factors that may stabilize the enzyme, and decreased degradation. In rats, nicotine has been shown to down-regulate select subtypes of ubiquitinating proteins in the hypothalamus and up-regulate these proteins in the prefrontal cortex (Kane *et al.*, 2004). Nicotine-induced changes in ubiquitinating proteins may influence CYP2D6 degradation by increasing or decreasing routing to degradation. Understanding the exact mechanism of brain CYP2D6 induction is an important direction for future studies, especially because this enzyme metabolizes a number of endogenous (e.g., neurotransmitters) and exogenous (e.g., neuroleptics) CNS-acting compounds.

As CYP2D6 is relatively uninducible in the liver, the ability to induce brain CYP2D6 would be beneficial in cases where low CYP2D6 levels may increase an individual's risk of disease, such as PD. The use of nicotine as a preventative measure in a high risk PD group would induce brain CYP2D6 to subsequently increase localized neurotoxin inactivation and increase neuroprotection. As a result, this may reduce the risk for PD or delay the onset of PD, especially since CYP2D6 is induced by nicotine in brain regions directly affected in the disease (e.g., the striatum and substantia nigra). The use of nicotine after PD on-set can provide symptomatic relief for patients (Kelton *et al.*, 2000; Vieregge *et al.*, 2001). As there is a loss of DA during PD, nicotine-induced brain CYP2D6 may help elevate DA levels from the metabolism of tyramine. Additionally, nicotine-induced brain CYP2D6 may assist in reducing further damage elicited by toxins that are inactived by CYP2D6.

Future studies should also determine if induced brain CYP2D6 results in a similar increase in CYP2D6 function, as the expression of a *functional* CYP protein in the brain is clinically more relevant. CYP2D6 activity can be tested *ex-vivo* using CYP2D6 probe substrates; however, this method has proven to be difficult as brain CYPs are more labile than

hepatic CYPs and show reduced activity with increased freezer storage time (Tyndale *et al.*, 1999; Voirol *et al.*, 2000). As in our recent *in situ* rat brain CYP2B study, we can utilize a CYP2D specific MBI, which essentially irreversibly binds to the enzyme upon its metabolism, to permanently label CYP2D in the brain. Using an animal model, the induced functional brain CYP2D levels *in situ* can be determined through the local CNS administration of a radiolabeled CYP2D specific MBI (**Table 5**) followed by the detection of immunoprecipitated radiolabeled brain CYP2D6 (Miksys & Tyndale, 2009). Paroxetine and MDMA are selective and potent MBIs of CYP2D6 that may be useful for measuring changes in functional brain CYP2D *in situ* (Bertelsen *et al.*, 2003; Van *et al.*, 2006; Yang *et al.*, 2006).

3.2 The influence of age on brain CYP2D6

CYP expression can undergo changes at different stages during development. For example, hepatic CYP3A7 expression predominantly occurs during prenatal stages of human life and is very low postnatally (Alcorn & McNamara, 2002; Hines, 2007). Conversely, hepatic CYP2D6 mRNA, protein and activity are fairly low in prenatal stages (Treluyer *et al.*, 1991; Alcorn & McNamara, 2002; Stevens *et al.*, 2008). Like the hepatic ontogenic studies, in **Chapter 3** we observed low levels of brain CYP2D6 expression prenatally that dramatically increased in postnatal stages (Treluyer *et al.*, 1991; Alcorn & McNamara, 2002; Stevens *et al.*, 2008). Changes at birth such as the loss of maternal repressors, stimulation by neonatal factors expressed at or after birth, and response to developmental changes and environment were suggested as causes for the surge in hepatic CYP2D6. These changes associated with birth may also affect CYP2D6 expression in the brain.

Hepatic CYP2D6 levels progressively increase in the first year of life to stable levels (Treluyer *et al.*, 1991; Alcorn & McNamara, 2002). Thus, the lack of change in brain CYP2D6 in the subsequent 19 years of life was expected. A surprising observation, however, was that

CYP2D6 increased throughout adulthood (20-80 years) in select brain regions. Considering that the principal function of CYP2D6 is the metabolism of drugs and toxins, the increase with age may be substrate upregulation where substrates are environmental components and/or CNS drugs. We reported that brain CYP2D is inducible in primates by nicotine, and human brain CYP2D6 is influenced by chronically abused drugs such as cigarettes and alcohol (Miksys *et al.*, 2002; Mann *et al.*, submitted). Furthermore, rat brain CYP2D can be induced by multiple CNS-acting drugs, including nicotine, clozapine, fluoxetine and thioridazine (Hedlund *et al.*, 1996; Haduch *et al.*, 2004; Haduch *et al.*, 2010). If one considers that, diagnosis and treatment of individuals for mental disorders (e.g., depression and schizophrenia) occur on average between the ages of 20 and 45 years, there is a likely increase in the consumption of CNS-acting drugs in adulthood that could influence CYP2D6 brain levels (Kessler *et al.*, 2007). Additionally, there is the increased likelihood of consumption, and co-use, of alcohol and tobacco during early adulthood, factors which are associated with higher brain CYP2D6 levels in humans (Miksys *et al.*, 2002; Mann *et al.*, submitted). In summary, one possible contributing factor to the CYP2D6 increase during adulthood is a response to clinical drugs and drugs of abuse and their accumulation over time, as well as other unknown environmental inducers. We were unable to test drug usage as a contributing factor to the age-related brain CYP2D6 increase, as we did not have drug histories for all of our control tissue donors.

Aging itself also directly affects all cells, tissues, and organs. Therefore, the increase in brain CYP2D6 during adulthood may also be a natural aging phenomenon. Age-dependent changes in DNA methylation patterns have been noted in various tissues, including the brain, and these changes are both organ- and brain region-specific (Hernandez *et al.*, 2010; Thompson *et al.*, 2010). The *CYP2D6* gene has potential methylation sites, therefore, age-

related epigenetic changes could alter brain CYP2D6 expression (Ingelman-Sundberg *et al.*, 2007).

Another possibility is that the increase in brain CYP2D6 may be a secondary response to other age-related CNS changes, such as the reduction in brain volume, white matter, myelinated fibers, and neuronal connections, and the overall loss of neurons (Piguet *et al.*, 2009; Fjell & Walhovd, 2010). As CYP2D6 can metabolize endogenous neurosteroids and neurotransmitters, the increase in brain CYP2D6 may be compensating for reduced neurotransmission that results from age-related neuronal damage (Kern & Behl, 2009). With increasing age and CNS damage, gliosis, a process of glial cell growth, replication and/or migration to a damaged area, is known to occur (Conde & Streit, 2006; Heneka *et al.*, 2010). We observed that in addition to astro-gliosis, both astrocytic CYP2D6 expression and the number of CYP-expressing astrocytes were higher in PD cases compared to controls (**Chapter 3**). This suggests that CYP2D6 likely increases during gliosis and/or CNS damage. Upon CNS damage, activation of microglia is the primary response, whereas astrocytes are the last cell population to be activated (Robel *et al.*, 2011). Even though we observed little to no astrocytic CYP2D6 expression in our PD age-matched controls, CYP2D6 may be increased in other glial cells (e.g., microglia), which we did not assess. Interleukin-2 can induce hepatic CYP2D in rats, suggesting that factors (e.g., interleukins) released during early gliosis *could* increase CYP2D6 expression in non-astrocytic cells (Kurokohchi *et al.*, 1992). Future studies should confirm which specific cells express CYP2D6 by co-labeling cells with CYP2D6 and markers for neurons (e.g. NeuN), oligodendrocytes (e.g. myelin-oligodendrocyte glycoprotein), and microglia (e.g. CD68) (Scolding *et al.*, 1989; Guillemin & Brew, 2004; Lind *et al.*, 2005). These studies can be extended to determine which cells show changes in CYP2D6 with age.

The effect of age on CYP2D6 expression was brain region specific. CYP2D6 increased with age in brain regions of both low (i.e., SN) and high (i.e., cerebellum) CYP2D6 expression. Notably, we observed that the hippocampus had relatively high CYP2D6 expression but was not influenced by age. This suggests that age affects CYP2D6 in regions with differing levels of CYP2D6 expression and further confirms the complexity of CYP2D6 regulation among brain regions.

CYP2D6 brain levels are influenced by factors such as genetic variation, age, brain region, and smoking. Unfortunately, due to the limited number of adult brain samples for each brain region (n=16 to 26 per brain region), we were unable to model the impact of each factor on CYP2D6 expression. When the effect of only age and genotype was assessed in the frontal cortex (n=76), age significantly influenced CYP2D6 expression and contributed substantially to the variation in this region. This supports the idea that regional expression of brain CYP2D6 is tightly regulated. More importantly, it indicates the necessity to use age-matched controls when assessing CYP2D6.

The SN shows a high degree of vulnerability to damage with aging and is a region of specific interest in PD (Rudow *et al.*, 2008). The progressive loss of dopaminergic neurons, although less than 1% per year, has been attributed to factors such as the accumulation of metals (e.g., iron) and the subsequent build up of toxins, the imbalance of autophagic recycling, increased oxidative stress, reduced efficiency of chaperones, and impaired responses to neurotrophic factors (Cherra & Chu, 2008; Migliore & Coppede, 2009; Hindle, 2010). In previous studies and in **Chapter 3**, we reported that human SN had low expression of CYP2D6 relative to other brain regions (Miksys *et al.*, 2002). This may reduce the ability of the SN to detoxify neurotoxins, which puts this region at greater risk of damage. We also showed that in the SN cellular expression CYP2D6 is increased with gliosis, which is believed to be a

protective response, in some cases, to control damage (Heneka *et al.*, 2010). Interestingly, CYP2D6 in this region is also highly responsive (i.e., inducible) to the neuroprotective agent nicotine and cigarette smoking which is associated with reduced risk for PD (Mann *et al.*, 2008). Collectively, increased CYP2D6 expression in the SN with age may be a protective mechanism in response to accumulating toxins, drugs, and/or signalling from age-related neurodegeneration processes.

3.3 The influence of Parkinson's disease on brain CYP2D6 expression

Neurodegeneration can be considered a result of an imbalance in toxicity promoting processes (i.e., ROS) and repair mechanisms. Prior to PD on-set the expression of certain proteins (e.g., over-expression of α-syn) and/or changes in protein function (e.g., CYP2D6) can either promote or reduce toxicity, altering an individuals risk for PD. During the course of PD there may also be changes in expression of select proteins, to repair and/or compensate for the damage that has occurred (e.g., neurotrophic factors).

In **Chapter 3** we showed that PD cases had lower CYP2D6 levels in the frontal cortex (~51%, P=0.001), cerebellum (~40%, P<0.001), and hippocampus (~47%, P>0.05) compared to age-matched controls. Whether these lower levels precede the disease or occur as a result of PD is difficult to assess in post-mortem human studies. The fact that lower levels of CYP2D6 were observed in regions not affected (i.e., cerebellum) or affected in later stages (i.e., frontal cortex) of PD would suggest that lower levels precede PD onset. This is additionally supported by the ~47% lower CYP2D6 levels in the hippocampus, which is a region affected in later stages of PD and a region where CYP2D6 appears to be unaltered by factors such as age, smoking, or nicotine. However, in regions directly affected in PD (the caudate and SN), which are more likely to undergo changes as a result of PD than unaffected regions, we observed levels of CYP2D6 which were similar between PD cases and controls. These results infer that

the lower levels in unaffected (i.e., cerebellum) or late-stage affected regions (i.e., frontal cortex) are representative of CYP2D6 levels prior to disease onset, while higher levels of CYP2D6 in affected regions may represent a response to damage in PD.

Lower CYP2D6 levels in PD cases were observed in brain regions which demonstrated both altered (e.g., cerebellum) and unaltered (e.g., hippocampus) CYP2D6 expression with increasing age. For example, both the frontal cortex and cerebellum which showed an increase in CYP2D6 expression with age had lower levels of CYP2D6 in PD cases compared to controls. Conversely, hippocampal CYP2D6 expression that showed no influence of age trended towards (~47%) lower levels of CYP2D6 in PD cases compared to controls. Thus, while age influenced the levels of brain CYP2D6 in select regions, the observed lower (~40–50%) CYP2D6 levels in the frontal cortex, hippocampus, and cerebellum of PD cases occurred in regions of both increase and no change in CYP2D6 with age. These results suggest that the lower levels in PD cases are unlikely to be a result of age-related changes in CYP2D6.

How CYP2D6 may change with age in PD cases is undetermined, however there are three possible interpretations: a) PD cases may have the exact same expression pattern from fetal to 80 years of age as non-PD cases, but CYP2D6 is ~40% lower than unaffected individuals, b) the expression profile is the same until early adulthood at which point CYP2D6 is less responsive to age-related increases in PD cases compared to non-PD cases, or c) PD cases at some point during their adulthood undergo a loss of CYP2D6 levels.

In our study, we predicted lower CYP2D6 levels in the SN and caudate of PD cases compared to controls. However, we observed no real differences between PD cases and controls using western blotting. This may be because in a diseased state, brain regions directly affected with the disease undergo various changes in an attempt to control, repair, and/or compensate for the damage (Kern & Behl, 2009; Hindle, 2010). Immune response activation

has been implicated in PD (Barnum & Tansey, 2010). An important element in CNS immune response is the activation of glia in response to damage (i.e., gliosis). In agreement with the literature, we noted larger and increased numbers of astrocytes that were more highly stained for GFAP in PD cases compared to controls, indicative of astrogliosis. We also observed more strongly labelled CYP2D6 positive astrocytes and intense CYP2D6 staining of other non-astrocytic cell types, that may be glial cells and/or neuronal cells, in PD cases compared to controls. As interleukin-2 can induce hepatic CYP2D in rat, inflammatory factors released during PD could potentially increase levels of brain CYP2D6 in PD cases (Kurokohchi *et al.*, 1992). Altogether, cellular changes that may occur due to damage in the SN and caudate are likely contributing to the lack of difference in CYP2D6 expression observed in these PD-affected regions by western blotting.

In rodent models of PD the SN, as well as the caudate and putamen, tend to accumulate neurotoxins, such as isoquinolines and MPP^+ (Herkenham *et al.*, 1991; Nagatsu, 1997; Lorenc-Koci *et al.*, 2006). This accumulation is thought to contribute to the selective neuronal degeneration in these brain regions. As a number of neurotoxins are substrates of CYP2D6 (e.g., isoquinolines), the higher than expected CYP2D6 levels in PD cases SN and caudate could also be to compensate for increased neurotoxic substrates in these regions. Substrates can induce their respective metabolizing CYP; the CYP2E1 substrate ethanol, for example, can induce both brain and hepatic CYP2E1 (Howard *et al.*, 2003; Joshi & Tyndale, 2006b). If CYP2D6 in the SN and striatum is increased in response to toxin-substrates, this would result in increased neurotoxin inactivation and thereby assist in reducing neuronal damage. In an attempt to better understand the role of CYP2D6 in the brain, future studies should isolate and identify distinct populations of cells within brain regions that express, or exhibit changes in, CYP2D6 with drug exposure, disease, or age.

4. Clinical relevance of altered brain CYP2D6 expression

Ex vivo studies demonstrate that brain CYP2D is functional and can metabolize a variety of CYP2D substrates including dextromethorphan and tyramine (Gilham *et al.*, 1997; Tyndale *et al.*, 1999; Voirol *et al.*, 2000; Haduch *et al.*, 2004; Bromek *et al.*, 2010). In **Chapter 1**, we showed that CYP2D6 in human neuroblastoma cells could metabolize the specific substrate AMMC, a reaction that was completely blocked by the selective CYP2D6 inhibitor quinidine. Similarly, SH-SY5Y cells in culture metabolized radiolabeled codeine to morphine, a reaction primarily mediated by CYP2D6 (Boettcher *et al.*, 2005). Another study demonstrated *in vivo* brain CYP2D metabolism by detecting significantly higher morphine levels in the brain 30 minutes after systemic injection of codeine, compared to the delivery of 1 mg/kg morphine given systemically; the dose of 1 mg/kg morphine i.p., resulted in equivalent plasma levels of morphine to 30 minutes after 20 mg/kg (i.p.) of codeine administration. The additional morphine detected in the brain was predicted to be due to local metabolism of codeine to morphine (Chen *et al.*, 1990). These studies suggest that CYP2D6 is functional in the brain, but more studies are required to determine *in situ* CYP2D6 metabolism.

Using a within animal design, our laboratory demonstrated that altering functional levels of CYP2B in the brain can affect the pharmacological response to the anaesthetic propofol (Khokhar & Tyndale, 2010). Inhibiting rat CYP2B in the brain with two distinct MBIs (C8-xanthate and 8-methoxypsoralen) given i.c.v., increased sleep time by propofol, which is metabolically inactivated by CYP2B. In contrast, rats treated with nicotine for 7 days, which induces rat brain but not hepatic CYP2B, had decreased sleep time that could be reversed by i.c.v., delivery of the CYP2B MBI. Together, these results suggest that altered levels of CYP in the brain can influence a drug's effect. We have shown that age, disease pathology, and select drugs can alter brain CYP2D6 levels. The significance of altered brain

CYP2D6 levels is that it can consequently influence local metabolism of CNS drugs, neurotransmitters, neurosteroids, and neurotoxins. This could alter drug response, behaviour, and degree of neurotoxicity which may alter risk for diseases like PD.

4.1 CNS Drug metabolism

The role of CYP2D6 in drug metabolism is important because it can metabolize ~30% of clinical drugs. This includes a variety of CNS-acting drugs, such as antipsychotics, antidepressants, analgesics, opioids, and anti-dementia drugs (**Table 1**) (Coutts & Urichuk, 1999; Kirchheiner *et al.*, 2004; Zanger *et al.*, 2004). Although CYP2D6 genetic variation has a large impact on interindividual variability in drug metabolism, there remains large variation in the degree of drug metabolism within a genotype (Ingelman-Sundberg *et al.*, 2007). It has been recognized that in some cases even when plasma levels are optimal, psychoactive drugs (e.g., neuroleptics and antidepressants) either do not elicit an effect or show adverse side effects (Michels & Marzuk, 1993b; a). Although the liver plays a large role in drug metabolism, localized CNS metabolism may also influence a drug's effect in the brain. The region-specific and highly localized expression of CYP2D6 in cells likely create metabolic microenvironments (Britto & Wedlund, 1992). Therefore, changes in CNS CYP2D6 expression may significantly influence localized drug metabolism and therefore CNS drug effect.

CYP2D6 metabolizes a number of antipsychotics, including risperidone, perphenazine, thioridazine, and haloperidol (Ingelman-Sundberg, 2005). One study found no correlation between plasma levels of risperidone, or its active metabolite, and drug response in schizophrenia patients (Spina *et al.*, 2001a). This supports the notion that plasma drug levels are not always good predictors of drug response and that altered CNS metabolism by CYP2D6 or other CYPs may influence a drug's effect. Interestingly, *CYP2D6* PM genotype is associated

with increased EPS, likely resulting from the inability to metabolize neuroleptics in the liver and/or brain (Schillevoort *et al.*, 2002; Tamminga *et al.*, 2003). Some studies show no correlation between drug dose or active metabolite plasma levels and EPS in patients treated with risperidone (Lane *et al.*, 2000; Riedel *et al.*, 2005). This suggests that differences in localized CNS metabolism may be contributing to this side effect. Although EPS are more common in individuals without a functional *CYP2D6* (PMs), they are also observed in *CYP2D6* EMs and we have shown that EMs have large variation in brain CYP2D6 expression (Coutts & Urichuk, 1999; Mann *et al.*, 2008; Mann *et al.*, submitted). This may contribute to altered drug response where low brain CYP2D6 expression would result in reduced localized inactivation of antipsychotics thereby increasing risk for neuroleptic induced EPS (Schillevoort *et al.*, 2002; Tamminga *et al.*, 2003).

The role of brain CYP2D6 in drug outcome is further supported by the observation that smokers display a lower prevalence for neuroleptic-induced parkinsonism and score lower on EPS scales than non-smokers (Jabs *et al.*, 2003). We have shown that smokers have higher levels of CYP2D6 in brain regions, especially the basal ganglia, involved in EPS (Jabs *et al.*, 2003; Mann *et al.*, 2008). Among other mechanisms, reduced EPS by smoking may in part be due to increased brain CYP2D6 and consequently increased localized CNS drug inactivation, especially as hepatic CYP2D6 is unaltered by smoking (Funck-Brentano *et al.*, 2005). Additionally, depressed smokers treated with a combination of selective serotonin reuptake inhibitors (substrates of CYP2D6) and a nAChR antagonist, had lower responses to the antidepressants compared to non-smokers (George *et al.*, 2008). The higher brain CYP2D6 in smokers may be responsible for reduced drug response in smokers because of an increased inactivation of antidepressants in the brain.

As brain CYP2D6 increases with age, the influence of localized CNS drug metabolism may be important in older patients. Older patients (>75 years) on the antidepressant drug desipramine, a CYP2D6 substrate, were less responsive than younger patients when controlling for drug dose and plasma levels (Nelson *et al.*, 1995). As 60 to 80 year-old individuals have high levels of brain CYP2D6, higher rates of localized CNS drug metabolism may contribute to the reduced response. Overall, the higher levels of brain CYP2D6 in individuals over 40 years of age, and in those exposed to nicotine or cigarette smoke, may render them more or less responsive to CNS drugs depending on whether the drug is activated (e.g., codeine) or inactivated (e.g., antidepressants) by CYP2D6, respectively. Conversely, lower levels of brain CYP2D6, such as those observed in PD cases, may increase the likelihood of negative side effects of CNS drugs at clinically optimal doses (e.g., antidepressants).

Future studies, using animal models, should determine how altered brain CYP2D levels may affect CNS drug metabolism *in situ*. Assessing response to CNS drugs (e.g., codeine), while manipulating functional levels of brain CYP2D, by induction or inhibition, would elucidate the influence of local brain CYP2D drug metabolism on CNS drug effects (e.g., analgesia). To locally inhibit brain CYP2D function the MBI propranolol, a β-adrenergic blocking agent, may be more useful than the antidepressant and potent CYP2D6 MBI paroxetine (as discussed in **Section 3.1**), as paroxetine has its own CNS effects (Rowland *et al.*, 1994; Bertelsen *et al.*, 2003). However, local CNS administration of propranolol may result in peripheral effects including the inhibition of hepatic CYP2D metabolism as it is highly lipophilic and can cross the BBB (Rowland *et al.*, 1994; Komura & Iwaki, 2005a). Identifying a non-CNS acting, potent, and selective MBI of CYP2D that does not cross the BBB would be useful for manipulating brain CYP2D *in vivo* and would help determine if brain CYP2D significantly influences CNS drug effect (e.g., codeine metabolism and analgesia),

neurotoxicity (e.g., 6-OHDA and MPTP), or neurotransmitter metabolism (e.g., DA and serotonin).

4.2 Endogenous CNS substrate metabolism

The expression of CYP2D6 in the brain is important because it can metabolize a number of endogenous compounds including monoamine-like substrates. Because CYP2D6 has brain region-specific localization and can metabolize DA, serotonin, epinephrine, norepinephrine, and tyramine, variation in CYP2D6 is thought to influence personality traits or mood (Yu *et al.*, 2003b; Wang *et al.*, 2009; Bromek *et al.*, 2010). Polymorphisms in *CYP2D6* are associated with differing mood and personality traits, and have been implicated in harm avoidance (Roberts *et al.*, 2004; Kirchheiner *et al.*, 2006; Gonzalez *et al.*, 2008; Ahlner *et al.*, 2010). Although studies are not consistent, *CYP2D6* PMs have been reported to be more anxiety prone, less social, and have a low competitive drive (Kirchheiner *et al.*, 2006; Gonzalez *et al.*, 2008).

In addition to neurotransmitters, CYP2D6 can metabolize other neuroactive compounds such as progesterone, allopregnanolone, and anandamide (Hiroi *et al.*, 2001; Kishimoto *et al.*, 2004; Niwa *et al.*, 2008; Snider *et al.*, 2008). Progesterone has been shown to modulate the synthesis and release of neurotransmitters and neuropeptides in response to stimuli (Pluchino *et al.*, 2006). In rats, progesterone was shown to increase serotonin turnover, catechol-*O*-methyltransferase activity, and MAO activity (Genazzani *et al.*, 2000). In humans, treatment with progesterone in women changes mood (Hlatky *et al.*, 2002). A metabolite of progesterone, allopregnanolone, can activate the γ-aminobutyric acid receptor, thus modulating mood, stress, and behaviour with anxiolytic, sedative, and antiepileptic effects (Pluchino *et al.*, 2006). Fluctuations in allopregnanolone have been related to psychological symptoms such as depression, anxiety, and irritability (Pluchino *et al.*, 2006). Anandamide is another neurosteroid

that is metabolized by CYP2D6 and is involved in mood, anxiety, and processing emotions. Anandamide is the endogenous form of cannabinoid and is an agonist to the cannabinoid-1 receptor that has been implicated in depression (Bambico & Gobbi, 2008). Altogether, variation in brain CYP2D6 expression could modulate levels of these neurosteroids and thereby effect mood (e.g., depression).

Another role for brain CYP2D6 may be in the synthesis of endogenous morphine. *In vivo* synthesis of morphine from tyramine and codeine was reported in the marine invertebrate *Mytilus edulis*, and this synthesis was significantly reduced by the administration of the selective CYP2D6 inhibitor quinidine (Zhu *et al.*, 2005; Zhu, 2008). These studies demonstrated that CYP2D6 is involved in endogenous morphine formation suggesting that CYP2D6 may be involved in nociception (Zhu, 2008). The thalamus is a brain region involved in nociception and a region with relatively high expression of CYP2D6 in primates (Mann *et al.*, 2008; Wilson *et al.*, 2008). The thalamus shows interindividual differences in activity that correlates with pain threshold (Dostrovsky, 2000; Lenz *et al.*, 2004; Ochsner *et al.*, 2006). Additionally, resting brain perfusion in the thalamus was shown to correlate with the *CYP2D6* genotype (Kirchheiner *et al.*, 2010). Collectively, it may be that the interindividual variation in nociception is in part related to differences in CYP2D6 expression and its ability to synthesize endogenous morphine.

The fact that CYP2D6 can metabolize so many important neurotransmitters and neuromodulators implies that CYP2D6 expression in the brain may be of significant clinical importance. However, there is a need to further understand the involvement of CYP2D6 in brain function. The existence of fully functional humans who lack any CYP2D6 activity (i.e., CYP2D6 PMs) indicates that CYP2D6 is not essential for brain function; however, because the PM genotype influences certain personality traits, CYP2D6 likely modulates

neurotransmitter metabolism. As these neuro-compounds are likely formed in the brain, alterations in brain CYP2D6 expression could potentially modulate levels of neurotransmitters and neuromodulators, thus leading to distinct personality traits, differences in pain thresholds, as well as alterations in mood.

4.3. Parkinson's disease

Some evidence suggests that PD is associated with a certain personality type (Benedetti *et al.*, 2000). PD cases are likely to be more introverted, inflexible, low novelty seeking, harm avoiding, more anxiety prone, and have quiet personality traits (Benedetti *et al.*, 2000; Kaasinen *et al.*, 2001). It is thought that the presence of these PD personality traits in early life may lead PD cases away from using substances that cause dependence and increase health risks, such as smoking, alcohol, and coffee, all which reportedly reduce the risk for PD (Ishihara & Brayne, 2006; Thacker *et al.*, 2007). Notably, there is some overlap of personality traits associated with PD and *CYP2D6* PM genotype. Both *CYP2D6* PMs (who are at increased risk for PD) and PD cases exhibit harm avoidance, are likely to be anxiety prone, and are less social (Roberts *et al.*, 2004; Kirchheiner *et al.*, 2006).

In addition to PD cases exhibiting certain personality traits, the co-morbidity of depression with PD also exists (Ishihara & Brayne, 2006). In a number of cases, depression is known to occur prior to PD and is a risk factor for PD (Lieberman, 2006). PD cases show lower levels of serotonin and notably, studies of depression with animal models indicate there is a disturbed metabolism of neurotransmitters, including serotonin and DA (Harvey, 2008; Krishnan & Nestler, 2010). Both serotonin and DA can be synthesized by CYP2D6 (Yu *et al.*, 2003b; Bromek *et al.*, 2010). We showed that PD cases have lower brain CYP2D6 expression compared to controls (**Chapter 3**). Therefore, these lower brain CYP2D6 levels may

contribute to the reduced serotonin and DA levels that consequently may lead to the premorbidity of depression in PD cases.

A number of studies show that *CYP2D6* PMs are over-represented in the PD population compared to controls (Deng *et al.*, 2004; Elbaz *et al.*, 2004; Duric *et al.*, 2007; Singh *et al.*, 2010). The highest incidence of *CYP2D6* PMs (10–15%) occurs in the Caucasian population (Gaedigk *et al.*, 2008). The most prevalent null allele in Caucasians is *CYP2D6*4,* which occurs at an allele frequency of ~25% (**Table 4**) and is commonly over-represented in the PD population (Armstrong *et al.*, 1992; Deng *et al.*, 2004; Gaedigk *et al.*, 2009). This result is similar to our observation in **Chapter 3**. *CYP2D6* UMs (*CYP2D6* gene copy number variants) are most prevalent (~20%) in North African (e.g., Ethiopia) and Middle Eastern populations (e.g., Saudi Arabia) (Ingelman-Sundberg *et al.*, 2007; Gaedigk *et al.*, 2008; Zhou, 2009). Notably, Caucasians have the highest occurrence of PD (**Figure 4**), and they have the highest prevalence of *CYP2D6* PM genotype (von Campenhausen *et al.*, 2005; Lix *et al.*, 2010). In contrast, ethnic populations with the highest prevalence of *CYP2D6* UM genotype have a much lower prevalence for PD. For example, the crude PD prevalence in North Africa and Saudi Arabia ranges from 7–40 per 100,000 compared to 130–1,400 per 100,000 in Europe (al Rajeh *et al.*, 1993; von Campenhausen *et al.*, 2005; Okubadejo *et al.*, 2006; Masalha *et al.*, 2010). In addition to differences in environmental exposures between the above mentioned populations, the striking concordance of *CYP2D6* phenotype with PD prevalence around the globe provides further support of a link between CYP2D6 and PD suggesting high CYP2D6 expression may be neuroprotective.

There have been reports indicating that CYP2D6 PMs are not associated with increased risk for PD (Bialecka *et al.*, 2007; Halling *et al.*, 2008). Our results suggest, however, that not only PMs, but also EMs with lower brain CYP2D6 expression may be at increased risk; this

observation may confound some of the epidemiological studies based exclusively on CYP2D6 genotyping and/or phenotyping. Given the large variation in brain CYP2D6 expression, perhaps PD risk assessment should not be determined by distinguishing individuals based on *CYP2D6* genotype grouping (i.e., PM *versus* EM) but rather by a continuum of CYP2D6 expression levels.

As CYP2D6 metabolizes plant alkaloids, pesticides, and neurotoxins, it is an ideal candidate to study as a gene-environmental factor that may influence PD risk (Ingelman-Sundberg, 2005). Assessing both gene and environmental factors has been observed to increase the degree of a gene effect on PD risk; an example is *SNCA* dinucleotide repeat sequence (*REP1*) variants within the promoter region (Gatto *et al.*, 2010). One variant *REP1 259*, which is protective, is not protective in the presence of pesticide exposure, while another variant *REP1 263* actually increases risk of PD which is augmented with exposure to pesticides. Therefore, future epidemiological studies should look specifically at environmental exposures and *CYP2D6* genotype in larger sample populations. These studies should also conduct a more detailed assessment of dose and duration of exposure to environmental toxicants, which may show a dose dependent and stronger association for CYP2D6 and PD risk.

Most studies of *CYP2D6* genotype and PD risk have suggested that *CYP2D6* genotype plays a small but significant role in PD risk (Christensen *et al.*, 1998; Deng *et al.*, 2004; Singh *et al.*, 2010). We show in **Chapter 2** and **Chapter 3** that brain CYP2D6 has a large variability in protein expression. Further, not only are individuals with *CYP2D6* null genotypes over-represented in the PD group, but EMs with PD had ~40-50% lower expression of brain CYP2D6 protein compared to control EMs. Thus low CYP2D6 expression may increase the risk of PD, while high levels may be protective. This is consistent with both our observation of increased toxicity by MPP^+ in cultured neurons after blocking CYP2D6 function (**Chapter 1**)

and another study demonstrating the over-expression of CYP2D6 in PC12 cells protected against MPP^+ cytotoxicity (Matoh *et al.*, 2003; Mann & Tyndale, 2010).

In the brain, CYP2D6 is expressed in the basal ganglia, astrocytes, glia, at the BBB, and in dopaminergic neurons (Gilham *et al.*, 1997; Miksys *et al.*, 2002; Dutheil *et al.*, 2010). This indicates that CYP2D6 is ideally situated to detoxify neurotoxins in the brain. Theoretically, if CYP2D6 in dopaminergic neurons is expressed at the terminal axons and positioned with the active site facing the extracellular fluid, CYP2D6 could inactivate toxins prior to their entrance into the neuron to elicit damage. Within cells, CYP2D6 is expressed on the plasma membrane as well as in mitochondria, and according to studies in hepatocytes, CYP2D6 is suggested to be expressed on the inner membrane of mitochondria (Miksys *et al.*, 2000b; Sangar *et al.*, 2009). As the mitochondria are a target site for most neurotoxins implicated in PD (e.g., rotenone and β-carbolines), CYP2D6 metabolism at this site makes it potentially an important player in neuroprotection (Miksys *et al.*, 2000b; Kotake *et al.*, 2007; Watabe & Nakaki, 2008; Burbulla *et al.*, 2010). Because CYP2D6 can inactivate various neurotoxins and is ideally situated in the brain, CYP2D6 is likely able to protect the brain from neurotoxic chemicals consequently reducing the risk for PD.

In cigarette smokers, high levels of brain CYP2D6 were observed, particularly in the basal ganglia including the SN. A number of studies indicate that smokers have a reduced risk for developing PD, and pre-treatment with nicotine can protect against many PD-causing neurotoxins *in vivo* and *in vitro* (Alves *et al.*, 2004; Khwaja *et al.*, 2007; Quik *et al.*, 2007c). In monkeys, pre-treatment with nicotine protected against nigrostriatal damage by MPTP (Quik *et al.*, 2006b). We have shown that nicotine can significantly induce monkey CYP2D in the basal ganglia and SN. Thus, the induction of brain CYP2D6 may be one mechanism by which smoking and nicotine protect against PD. These observations, taken together with the

knowledge that *CYP2D6* PMs are at increased risk for PD, support the idea that lower CYP2D6 expression in individuals is likely contributing to the increased risk for PD, while higher CYP2D6 levels reduce this risk.

In regions directly affected in PD (i.e., the caudate and SN), we observed higher CYP2D6 expression than expected compared to unaffected brain regions. Since, CYP2D6 can synthesize DA and is ideally situated in dopaminergic neurons, these higher levels of CYP2D6 in the basal ganglia may be a mechanism to compensate for the loss of DA during PD (Gilham *et al.*, 1997; Riedl *et al.*, 1999; Bromek *et al.*, 2010; Gonzalez-Hernandez *et al.*, 2010). Nicotine treatment has been shown to have direct effects on dopaminergic tone and improve motor symptoms in PD cases, where nicotine-induced brain CYP2D6 may assist in the improvement of symptoms by increasing the synthesis of DA (Fagerstrom *et al.*, 1994; Kelton *et al.*, 2000; Quik *et al.*, 2007b). These induced CYP2D6 levels would also help alleviate some of the toxic burden of endogenous and exogenous neurotoxins and reduce further damage caused by PD-neurotoxins.

The caudate, putamen and SN experience a high degree of oxidative stress (Jenner & Olanow, 1996; Gonzalez-Hernandez *et al.*, 2010; Surmeier *et al.*, 2010). NO is a contributor to ROS and is implicated in PD pathogenesis (Jenner & Olanow, 1996; Tieu *et al.*, 2003). A high content of peroxides in these sensitive brain regions also promotes ROS and oxidative damage (Jenner & Olanow, 1996; Olanow, 2007; Gonzalez-Hernandez *et al.*, 2010). Recently, it was suggested that CYP2D6 likely metabolizes NO in the brain (Hall *et al.*, 2009). Also notable is the fact that CYP2D6 can use hydrogen peroxide (H_2O_2) as an oxygen donor source to metabolize substrates (peroxide shunting) (Julsing *et al.*, 2008). Together, these results suggest that CYP2D6 may not only neuroprotect by detoxifying potential PD-neurotoxins, but also by utilizing and reducing components that directly contribute to ROS formation. As mentioned

previously, substrates can induce their respective CYPs and NO is shown to regulate hepatic CYP2D6 *in vitro* (Hara & Adachi, 2002; Howard *et al.*, 2003; Haduch *et al.*, 2010). Therefore, the increase of NO and/or peroxides in this region may also induce CYP2D6 expression, as a mechanism of cellular defence to neuroprotect against ROS mediated damage.

Future studies should identify high-risk populations and place them on a nicotine transdermal patch to determine if nicotine alone can delay the onset of PD. Caucasians would be a population group of interest, particularly members of this group who are regularly exposed to pesticides, have a family history of PD, suffer from depression, struggle with sleep disorders, or have problems with olfaction, all which are risk factors for PD (Buck *et al.*, 2010; Tanner, 2010; Vance *et al.*, 2010). In terms of CYP2D6 research, future studies should use neuroimaging techniques (e.g., positron emission tomography) to detect CYP2D6 with radioisotope labelled MBIs (e.g., paroxetine) or competitive inhibitors (e.g., quinidine) and measure levels of brain CYP2D6 in PD high-risk groups.

Another avenue for future studies is to identify tissues that can easily be assessed and are good predictors of general brain CYP2D6 levels. For example, platelet MAO activity is often used as a representative of brain MAO levels (Berggren *et al.*, 2000; Husain *et al.*, 2009). White blood cells may be useful to assess representative brain CYP2D6 activity, as they demonstrate CYP2D6-specific codeine metabolism to morphine (Zhu *et al.*, 2005; Zhu, 2008). Conducting a correlational analysis with neuroimaging of functional brain CYP2D6 and white blood cell CYP2D6 activity could be used to validate the predictive value of white blood cells. Another option is to test cerebral spinal fluid, since previous studies have used this to measure levels of neurotransmitter metabolism in the brain (Fairbanks *et al.*, 1999). Further, levels of neurotransmitter in cerebral spinal fluid have been shown to correlate with behaviour and mental disorders (Fairbanks *et al.*, 1999; Sher *et al.*, 2006). Testing cerebral spinal fluid for

changes in CYP2D6-specific substrate metabolites (post-treatment) may be useful in assessing brain CYP2D6 activity. This could be further extended to measure changes in metabolites of CYP2D6-specific drugs or neurotransmitters upon administering a potent inhibitor of CYP2D6 (e.g., quinidine or the MBI paroxetine).

A question that remains to be answered is how important the localized expression of CYP2D6 is in terms of protecting or influencing the risk for PD. Using a CYP2D6 MBI or inducer in an *in vivo* model of PD (e.g., MPTP) to determine how altered brain levels of CYP2D6 can influence neurotoxic effects would be useful. Nicotine could be used as a brain CYP2D6 inducer; however, because nicotine is neuroprotective it would be difficult to distinguish the neuroprotective effects of induced brain CYP2D6 from those of nicotine. Instead, localized CNS expression of CYP2D6 could be increased by CNS delivery of the *CYP2D6* gene using an adeno-associated viral vector, which is an efficient vehicle used for gene transfer into the brain (Feng & Maguire-Zeiss, 2010; Van der Perren *et al.*, 2011). Fluorescently tagged CYP2D6 could be transfected into the CNS, as this would allow for non-invasive detection of gene expression while allowing one to monitor changes in CYP2D6 expression in response to neurotoxin treatment.

Altogether, CYP2D6 expression in the brain is important as it can metabolize a variety of CNS acting substrates including neurotoxins, CNS drugs, neurotransmitters, and neurosteroids. There is large interindividual variability in brain CYP2D6 expression; furthermore the expression is affected by smoking, nicotine, age and disease state. This variation in brain CYP2D6 expression may therefore influence an individual's ability to locally metabolize CNS acting substrates. Ultimately, these differences would result in individuals being more or less sensitive to neurotoxins, patients responding differently to CNS drugs, and individuals having differing personalities.

Conclusions:

In conclusion we found that, a) inhibiting CYP2D6 in neuronal cultures increases MPP^+ induced neurotoxicity, b) smoking, which reduces the risk for PD, is associated with higher brain CYP2D6 levels in humans, c) the neuroprotective agent nicotine can induce primate CYP2D in select brain regions, especially in the basal ganglia and SN, d) CYP2D6 expression increases with age during adulthood in select brain regions, and e) PD cases have ~40% lower expression of brain CYP2D6 compared to age-matched controls.

Lower brain CYP2D6 levels in individuals may compromise their ability to inactivate PD-causing neurotoxins, thereby increasing their risk for PD. Conversely, high or induced brain CYP2D6 may result in increased neurotoxin inactivation and reduced neuronal damage thereby decreasing PD risk. Additionally, altered levels of brain CYP2D6 may significantly influence localized metabolism of CNS drugs, drugs of abuse, and endogenous CNS compounds. Future studies should a) elucidate the mechanism of induction and regulation of brain CYP2D6, b) discover a useful clinical measure or predictor of brain CYP2D6 expression and function in humans, c) address whether variation in brain CYP2D6 expression can influence the required dosage of psychoactive drugs or alter neurotransmitter levels, and d) determine if direct inhibition or induction of CNS CYP2D6 can modulate drug response, behaviour, and/or neurotoxicity.

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