The Effect of Altering Brain CYP2B Activity on Nicotine Self-Administration Behaviour and Nicotine Levels in the Brain

by

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Abstract

Cytochrome P450 (CYP) enzymes play an important role in drug metabolism. While CYPs are abundantly expressed in the liver, where CYP-mediated drug metabolism typically occurs, these enzymes are also expressed in other tissues such as the brain. Local brain drug metabolism can influence the response to drugs that act within the brain. The CYP subfamily 2B (CYP2B) is expressed in the brain and is responsible for metabolizing many central nervous system (CNS)-acting drugs including nicotine, the main psychoactive ingredient in cigarettes. Genetic variation in human *CYP2B6* is associated with greater conversion to nicotine dependence and risk of relapse in smokers without influencing peripheral nicotine metabolism. This suggests that local brain nicotine metabolism could influence nicotine levels that in turn mediate nicotine reinforcement and resulting behaviours. The role of brain CYP2B activity in nicotine reinforcement was investigated by injecting a pharmacological CYP2B inhibitor into the brain of rats that then underwent nicotine self-administration (NSA), which models smoking behaviour. Inhibitor-treatment increased NSA acquisition, motivation to obtain nicotine and the number of sessions required to extinguish behaviour, suggesting that inhibiting brain CYP2B activity can augment nicotine-reinforced behaviour. The effect of brain CYP2B inhibition on brain nicotine levels following a nicotine injection was then investigated using *in vivo* microdialysis. Inhibitor

treatment increased peak brain nicotine levels and nicotine levels within 0-45 minutes post-injection compared to vehicle, suggesting that inhibiting brain CYP2B activity reduced nicotine metabolism, resulting in higher brain nicotine levels. Inducing brain CYP2B, by a paradigm that increases CYP2B protein in the brain but not the liver, reduced brain nicotine levels within 15-45 minutes post-injection, suggesting that induction increased brain CYP2B activity and nicotine metabolism. These findings demonstrate that altering brain CYP2B activity can influence brain nicotine levels and that inhibiting brain CYP2B, which increases nicotine levels, may increase nicotine's reinforcing effect consistent with the behaviours in NSA. This is also consistent with the behavioural differences associated with genetic variation in *CYP2B6*, suggesting that altered brain CYP2B activity may influence smoking behaviour. Thus, these findings provide evidence that brain CYPs can influence the local metabolism of its substrates and their resulting drug response.

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List of Abbreviations

CYP	Cytochromes P450
NSA	Nicotine self-administration
COPD	Chronic obstructive pulmonary disease
PR	Progressive ratio
CSF	Cerebrospinal fluid
CNS	Central nervous system
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
IC	Intracerebral
ICV	Intracerebroventricular
BDE-47	2,2',4,4'-tetrabromodiphenyl ether
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
DNA	Dioxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
PB	Phenobarbital
PBREM	Phenobarbital response element
CAR	Constitutive androstane receptor
PXR	Pregnane X receptor
MBI	Mechanism-based inhibitor
8-MOP	8-methoxypsoralen
GABA	Gamma-aminobutyric acid
PET	Positron emission tomography
C8X	C8-xanthate
^{3}H-8-MOP	^{3}H-8-methoxypsoralen
FMO	Flavin-containing monooxygenase
UGT	Uridine diphosphate-glucuronosyltransferase
nAChR	Nicotinic acetylcholine receptor
VTA	Ventral tegmental area
NMDA	N-methyl-D-aspartate
FR	Fixed ratio
IV	Intravenous
SC	Subcutaneous
PROD	7-pentoxyresorufin-O-dealkylation
IG	Intragastric
ACSF	Artificial cerebrospinal fluid
LC-MS	Liquid chromatography mass spectrometry
HPLC	High performance liquid chromatography
FT	Food training
SP	Spontaneous acquisition
ANOVA	Analysis of variance
SEM	Standard error of the mean
6-OHDA	6-hydroxydopamine
ICSS	Intra-cranial self-stimulation
CPP	Conditioned place preference
LTP	Long term potentiation
EPSC	Excitatory postsynaptic current

IPSC	Inhibitory postsynaptic current
siRNA	Small interfering ribonucleic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CITGO	6-(4-chlorophenyl)-imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime
MPP+	1-methyl-4-phenylpyridinium ion
AMPK	Adenosine monophosphate-activated protein kinase alpha
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
PDE	Phosphodiesterase
AKAP	A kinase anchoring proteins
3α, 5α-THP	5α-pregnan-3α-ol-20-one
RXR	Retinoid X receptor
PPARα	Peroxisome proliferator-activated receptor α
OEA	Oleoylethanolamide
PEA	Palmitoylethanolamide
NURR1	Nuclear receptor related 1
DR	Direct repeat
NBRE	Nerve growth factor IB response element
NGFI-B	Nerve growth factor IB
NurRE	Nur response element
SSRI	Selective serotonin re-uptake inhibitor

Section 1: Introduction

Statement of Problem

Tobacco dependence has been a health issue for decades, persisting even after the first report that smoking tobacco cigarettes was detrimental to health by the U.S. Surgeon General (USDHHS, 1964). It is well-established that smoking is addictive and that long-term smoking can lead to several health consequences that include reduced life expectancy and increased risk for developing lung cancer, chronic obstructive pulmonary disorder (COPD), heart disease and stroke (Fagerstrom, 2002). Despite this, almost one billion people worldwide (WHO, 2012) and approximately 16% of Canadians (CTUMS, 2012) are current smokers. Furthermore, a survey of Canadians in 2013 reported that approximately 50% of smokers attempted to quit for a 24 hour period of time within the past year, with only 11% of these smokers remaining abstinent (Reid, 2015). These data indicate that there is still a substantial proportion of smokers who remain dependent on tobacco, suggesting that populations of increased risk for dependence may exist among smokers. Smoking has a heritability component, where the genetic contribution to initiating and persisting in smoking behaviour has been estimated between 40-60% from twin studies (Li et al., 2003). Genes that have been associated with smoking include those involved in the pharmacokinetics (CYP enzymes) and pharmacodynamics (nicotinic acetylcholine and dopamine receptors) of nicotine (Al Koudsi and Tyndale, 2005), the main psychoactive ingredient in tobacco cigarettes (Schmeltz and Hoffmann, 1977; Benowitz et al., 1983). This suggests that variation in the expression of these genes modulates tobacco use and subsequently dependence.

CYP enzymes are responsible for metabolizing many drugs and endogenous compounds. Nicotine undergoes CYP-mediated metabolism, where it is primarily metabolized by CYP2A6 (Nakajima et al., 1996b) with a secondary contribution by CYP2B6 in the liver (Yamazaki, 1999). Although systemic levels of drugs that are CYP substrates, and their metabolites, are largely mediated by the activity of these enzymes in the liver, drug response does not always correlate with drug or metabolite plasma levels (Michels, 1993). It is possible that CYPs expressed in the organs where their substrates exert their actions may contribute to local drug levels and resulting drug response. Brain CYPs are expressed in various regions throughout the

brain (Ferguson, 2011), and can metabolize the same substrates as CYPs in the liver when assessed *in vitro* (Miksys and Tyndale, 2002) and *in vivo* (Miksys and Tyndale, 2009). Many CNS-acting drugs are CYP substrates; therefore CYPs expressed in the brain could influence drug levels in this organ, independent of CYP expression in the liver. Nicotine mediates its reinforcing effects in the brain (Henningfield and Keenan, 1993; Kenny and Markou, 2006); thus if local brain CYP activity can modulate brain nicotine levels, it could influence the processes mediating dependence. Altering CYP activity in the brain can alter brain, but not plasma, drug levels and subsequent response to drugs that are CYP substrates, such as the anaesthetic propofol, the pesticide chlorpyrifos, and the analgesic codeine (Khokhar, 2011; Khokhar, 2012; Zhou, 2013; McMillan and Tyndale, 2015). These findings are the first to suggest that variation in brain CYP activity can impact drug pharmacokinetics in the brain enough to change drug response.

Smoking cessation studies in humans suggest that *CYP2B6* genotype can influence smoking behaviours (Lee et al., 2007a; Lerman et al., 2007); however, peripheral nicotine levels between smokers who are *CYP2B6* slow vs. normal metabolizers are not different (Lee et al., 2007b). Both CYP2A6 and CYP2B6 are expressed in the liver; however, only CYP2B6 protein has been previously detected in the brain (Miksys and Tyndale, 2004), suggesting that CYP2B6 could play a relatively greater role in nicotine metabolism in the brain. Therefore it is possible that genetic variation in *CYP2B6* expressed in the brain may alter the levels of nicotine specifically in the brain without affecting the liver. This variation in brain nicotine levels may then contribute to the smoking behaviour differences seen between slow and normal *CYP2B6* metabolizers. It is unknown if variation in brain CYP2B6 activity can alter nicotine levels in the brain and/or if differences in nicotine brain levels are sufficient to change nicotine-reinforced behaviours.

Purpose of the Study and Objective(s)

The purpose of this work is to investigate a possible role for brain CYP2B in brain nicotine metabolism and in smoking behaviour using the nicotine self-administration paradigm. Using a rat model where we can alter the rat CYP2B enzyme, the homolog to human CYP2B6, specifically in the brain, we can test the effect of altered CYP2B activity in the brain on brain nicotine levels and on nicotine self-administration behaviours.

Statement of Research Hypotheses and Rationale for Hypotheses

1. Effect of operant food training and training dose of nicotine on intravenous nicotine self-administration

Nicotine self-administration is a commonly used model of smoking behaviour. Different parameters of study design exist for this model that can influence performance. Important parameters include the infusion dose of nicotine, prior food training, session length, access to the operant box (continual daily use or intermittent five day use), response operandum (lever vs. nose-poke) and use of different auditory or visual cues (Donny et al., 1998; Chaudhri et al., 2006; Clemens et al., 2010). Therefore, before testing the effect of altered CYP2B activity in this model, the parameters by which this effect can be discerned in acquisition, the progressive ratio (PR) schedule, and nicotine-seeking behaviour are important to determine. Prior training for food responding and infusion dose are two parameters that can affect acquisition and responding in nicotine self-administration. Food training is known to facilitate acquisition but have no effect on nicotine-seeking behaviour (Clemens et al., 2010) and higher infusion doses of nicotine are known to increase acquisition, motivation, and nicotine-seeking behaviour (Shoaib et al., 1997; Shram et al., 2008a); however the effect of food training on nicotine's dose-response curve for acquisition has not been fully characterized. Determining these parameters will allow us to decide when food training will be useful to train animals and which nicotine doses should be used in order to measure the effect of altering brain CYP2B activity.

Hypotheses: Prior food training will result in (1) more animals acquiring nicotine self-administration behaviour, and (2) greater motivation to obtain nicotine in the PR schedule of reinforcement which measures motivation, but (3) have no effect on nicotine seeking behaviour during extinction and reinstatement compared to no prior food training (spontaneous acquisition) at the same infusion doses.

2. Effect of brain CYP2B inhibition on intravenous nicotine self-administration behaviour

If brain CYP2B activity can influence brain levels of nicotine independently from liver CYP2B activity, than reducing CYP2B activity in the brain using CYP2B inhibitors is expected to reduce nicotine metabolism within the brain. If less nicotine is metabolized than more nicotine may be available in the brain to exert its actions on central nicotinic acetylcholine receptors. Dopamine release is believed to mediate the reinforcing effect of drugs of abuse including nicotine (Di

Chiara and Imperato, 1988) and this dopamine release increases dose-dependently with increasing nicotine doses (Di Chiara and Imperato, 1988; Marshall et al., 1997); therefore, greater nicotine available from brain metabolism inhibition may increase the reinforcing effect of nicotine at a given nicotine dose. Greater nicotine reinforcement would increase nicotine self-administration behaviour, as this paradigm exhibits dose-dependent behavioural responses in acquisition, PR responding, extinction and reinstatement (Corrigall and Coen, 1989; Shaham et al., 1997; Donny et al., 1998; Donny et al., 1999).

Hypotheses: Inhibiting brain CYP2B is hypothesized to increase (1) acquisition, (2) motivation to respond for nicotine during the PR schedule of reinforcement, and (3) nicotine-seeking behaviour during extinction and reinstatement.

3. Effect of brain CYP2B inhibition and induction on nicotine brain levels measured by *in vivo* microdialysis

If inhibitor treatment within the brain can influence nicotine self-administration behaviour then we would expect to see greater levels of nicotine in the brain and not in the blood. This would suggest that the change in behaviour is mediated through variation in brain CYP2B activity. Greater nicotine levels in the brain, after inhibiting brain CYP2B, would increase the amount of nicotine circulating through the cerebrospinal fluid (CSF), as this is considered the amount of free levels available in the brain (Liu et al., 2009). Therefore, we would expect greater nicotine levels measured from CSF in the lateral ventricles in rats pre-treated centrally with inhibitor following peripheral nicotine administration. In conjunction with greater nicotine levels in the brain, if less cotinine is produced by CYP2B in the brain, we would expect lower cotinine levels in the CSF. The opposite would be expected in rats following induction of brain CYP2B. Brain CYP2B induction would result in more CYP2B activity, which would increase nicotine metabolism in the brain. Thus lower nicotine and higher cotinine levels would be expected in the CSF.

Hypotheses: Following 7-day nicotine treatment, (1) brain nicotine levels will be lower while brain cotinine levels will be higher following peripheral nicotine administration in rats. If CYP2B induction is responsible for reduction in nicotine brain levels, (2) inhibiting CYP2B activity in the brain after 7-day nicotine treatment will reverse this effect, increasing brain nicotine levels and decreasing brain cotinine levels. (3) Repeatedly administering the CYP2B

inhibitor into the brain during the 7-day nicotine treatment will also increase brain nicotine levels and decrease brain cotinine levels after peripheral administration of nicotine.

Review of the Literature

1 Cytochrome P450 enzymes

The CYP families 1-3 are primarily responsible for metabolizing drugs and other xenobiotic compounds (Nebert and Russell, 2002; Ferguson and Tyndale, 2011). These enzymes carry out phase I drug metabolism which involves the biotransformation of substrates into polar or hydrophilic compounds. Biotransformation is commonly mediated through oxidation of the substrate; however, CYPs can undergo multiple reactions such as reduction, hydrolysis, ester cleavage, ring formation, dehydration, coupling reactions, phospholipase activity (Guengerich, 2001). These reactions can inactivate a drug, activate a compound, or produce a metabolite with different pharmacology from the substrate. Drug metabolism occurs mainly in the liver where these CYPs are highly expressed and this hepatic metabolism is largely responsible for systemic concentrations of the drug and its metabolites.

Although the liver is the major site for drug metabolism, a growing body of evidence suggests that extra-hepatic drug metabolism can also occur and significantly influence drug response. CYPs are also expressed in other tissues such as the gastrointestinal and respiratory systems (Ding and Kaminsky, 2003), and the central nervous system (Ghersi-Egea et al., 1993; Bhagwat et al., 1995). Expression of CYPs at the site of action of their substrates or metabolites could result in local drug metabolism independent of hepatic metabolism, possibly altering local drug and metabolite levels and potentially influencing drug effects.

Many CNS-acting drugs are CYP substrates, therefore variation in local drug metabolism could, in part, explain why systemic levels of CNS drugs do not always correlate with drug response (Michels, 1993). CYP expression can vary by genetics and/or by exposure to compounds from the environment (Zanger and Schwab, 2013); there is variation in the genes encoding drug-metabolizing CYPs and many of these CYPs can also be induced or inhibited by xenobiotics

either in multiple tissues (Volk et al., 1995; Schilter et al., 2000) or specifically in the brain (Miksys et al., 2000a). Variation in brain CYP metabolism would alter brain drug levels, affecting, in turn, drug efficacy or safety. This could have important clinical consequences as the delivery of enough drug concentration to produce therapeutic levels, neurotoxicity or off-target effects in the brain are major issues with CNS-acting drugs. Clinical development of new CNS drugs in the pharmaceutical industry takes longer to develop than non-CNS drugs (Pangalos et al., 2007); understanding factors that can influence brain drug levels, such as the role of altered drug metabolism in the brain, might be useful both in developing new drugs and in modifying administration for existing drugs on the market.

1.1 CYPs in the brain

1.1.1 CNS-acting drug substrates

CYP families 1-3, responsible for metabolizing many CNS drugs, are expressed throughout the brain. CYP1A and CYP1B are known for activating carcinogenic polycyclic aromatic hydrocarbons (Nebert et al., 2004) but they are also involved in the metabolism of centrally-acting drugs, such as anti-depressants and anti-psychotic drugs as well as endogenous steroids such as estrogen (Lewis et al., 2004).

The CYP2 family is the largest family of drug metabolizing CYPs, of which CYP2B, CYP2C, CYP2D and CYP2E subfamilies are found in the brain (McFadyen et al., 1998). For CNS-acting drugs, CYP2B, CYP2C, and CYP2D forms can metabolize anti-depressants, while CYP2B, CYP2D and CYP2E1 can metabolize drugs of abuse (Lewis et al., 2004) as well as toxins (Gut et al., 1996; Gilham et al., 1997; Tang, 2001), and CYP2D can metabolize opioid analgesics (Gudin, 2012). CYP2B and CYP2D can also metabolize endogenous compounds that act in the brain: CYP2B can metabolize steroids such as testosterone (Rosenbrock et al., 1999), and CYP2D can metabolize the neurotransmitters dopamine (Bromek et al., 2011) and serotonin (Haduch et al., 2015).

In the CYP3 family, CYP3A4 metabolizes the vast majority of drugs. CYP3A4 can metabolize CNS-acting anti-depressants, barbiturates, opioid analgesics and hypnotics, in addition to non-CNS-acting drugs (Li et al., 1995; Frye, 2004). Therefore, its expression in the brain has the potential to influence not only the effects of CNS-acting drugs where their primary action is in

the brain, but also to influence off-target effects of non-CNS-acting drugs that may cross the blood brain barrier. Many CNS-acting drugs are substrates for these CYP1-3 isoforms expressed in the brain, suggesting the possibility of metabolism near the site of action within the brain.

1.1.2 Expression in the brain

CYP1-3 isoforms are expressed in a region and cell-specific manner within the brain (Anandatheerthavarada et al., 1990; Farin and Omiecinski, 1993; Schilter and Omiecinski, 1993; McFadyen et al., 1998; Meyer et al., 2007; Toselli et al., 2015b). This heterogeneity in expression is present in different species, including humans, non-human primates, and rodents. In rodents total brain CYPs represent about 1-4% the amount found in the liver (Sasame et al., 1977; Warner et al., 1988); however, in some cases the expression within some cell types can be as high as or higher than that in hepatocytes (Miksys, 2000). CNS drugs usually act on specific neurotransmitter systems or receptor types in the brain, which suggests that the regional diversity of CYP brain expression may have a specific role in detoxification to protect cell function and that brain CYPs may play a role in the local metabolism of their substrates within certain brain regions.

In humans, CYP1A mRNA and protein are expressed in the cortex, thalamus, and within brain stem nuclei including the substantia nigra, red nucleus, pons, median raphe and locus coeruleus (Farin and Omiecinski, 1993; McFadyen et al., 1998). CYP1A activity has been reported from mitochondrial fractions of the olfactory bulb in monkey brain, suggesting it is expressed in the brain of this species (Iscan et al., 1990). CYP1A mRNA and protein are expressed in rat cortex, cerebellum, midbrain structures, and striatum (Schilter and Omiecinski, 1993), and in mouse cortex and hippocampus (Stamou et al., 2014). CYP1B mRNA and protein are expressed in neurons and astrocytes from human cortical tissue and the putamen (Rieder et al., 1998; Rieder et al., 2000; Muskhelishvili et al., 2001). Given the role of these enzymes in the bio-activation of procarcinogens, their expression in the brain could have negative consequences if the brain is exposed to these compounds.

The CYP2 isoforms are also expressed heterogeneously throughout the brain. CYP2B6 mRNA and protein, the CYP2B isoform found in humans and monkeys, is detected in the cortex, midbrain structures such as the striatum, hippocampus and basal ganglia, and cerebellum in humans (Gervot et al., 1999; Miksys, 2003) and in monkeys (Lee et al., 2006a). Rat CYP2B

mRNA and protein are also expressed in these regions, in addition to the olfactory bulbs and hypothalamus (Schilter and Omiecinski, 1993; Miksys, 2000). Fewer studies have examined CYP2B expression in mouse brain; however, mRNA has been detected in whole brain (Renaud et al., 2011) and in cortical and hippocampal tissue (Stamou et al., 2014). Human CYP2C mRNA and protein, specifically the isoforms CYP2C9 and CYP2C19, are expressed in the frontal cortex, midbrain structures such as the hippocampus, amygdala and basal ganglia, and cerebellum (McFadyen et al., 1998; Booth Depaz et al., 2015). In rat brain, CYP2C13 protein is expressed in similar regions as human CYP2C (Riedl et al., 2000). Human CYP2D isoform CYP2D6 mRNA and protein are expressed in cortex, midbrain, brainstem, and cerebellum (Miksys, 2002; Dutheil et al., 2009). In mouse (Miksys et al., 2005), rat (Miksys et al., 2000b) and monkey brain (Mann et al., 2008), CYP2D protein is expressed in similar brain regions to human CYP2D6. CYP2E1 mRNA and protein are expressed in the cerebellum, cortical tissue and hippocampus of both humans and rats (Upadhya et al., 2000; Howard et al., 2003; Toselli et al., 2015a), and a similar pattern of protein expression has been reported in monkeys (Joshi and Tyndale, 2006b). In conclusion, the CYP2 family as a whole has widespread expression throughout the brain in multiple species, which suggests that its members may be important in the biotransformation of drugs, toxins, and endogenous compounds that act on the brain.

CYP3A4 mRNA has been reported in the frontal cortex and basal ganglia of humans (McFadyen et al., 1998); however more recent studies report low or undetectable mRNA levels in human brain (Dutheil et al., 2009; Toselli et al., 2015b). CYP3A4 protein is detected in human brain (Booth Depaz et al., 2013). Rat brain expresses CYP3A1 mRNA (Schilter and Omiecinski, 1993; Pai et al., 2002), and mouse brain expresses CYP3A11 and CYP3A13 mRNA and protein (Rosenbrock et al., 2001b; Hagemeyer et al., 2003). As this subfamily is involved in metabolizing the majority of clinically prescribed drugs, its presence in the brain may influence their effects within the brain.

Together, many drug metabolizing CYP enzymes are expressed in the brain, which suggests that they might deactivate/activate or detoxify/toxify compounds at the local site of action. This might be a strategy to prevent insults from xenobiotic compounds in such a critical organ as the brain, where damage might have major consequences. However, metabolism by CYPs might have the opposite effects where they can activate toxic compounds that would elicit damage. Thus, the role of CYP activity in the brain may be important for drug efficacy and safety.

1.1.3 CYP activity in the brain

Expression of drug metabolizing CYPs in the brain is not sufficient evidence to demonstrate the existence of local drug metabolism in this organ. CYP-mediated metabolism involves the participation of other proteins that mediate electron transfer to reduce the iron component of CYPs in order to change the substrate, thus the presence of these proteins, called coenzymes, in the brain would be required if brain CYPs are active. The coenzyme necessary for CYPs to carry out their reactions with substrates, NADPH oxidoreductase, is expressed in the brain (Ravindranath et al., 1990; Norris et al., 1994), supporting potential brain CYP metabolic activity.

Early studies investigating brain CYP activity *in vitro* reported activity using subcellular brain fractions. Both human (Ravindranath et al., 1989) and rat (Ravindranath et al., 1990) brain microsomes were capable of metabolizing morphine, ethoxycoumarin and aminopyrine, and human brain mitochondrial preparations were also found to metabolize ethoxycoumarin and aminopyrine (Bhagwat et al., 2000). The later substrate was also found to metabolize mouse brain microsomes (Ravindranath and Anandatheerthavarada, 1989). Metabolism by specific CYP isoforms in the brain has also been characterized in rodents *in vitro*. CYP2D was found to metabolize dextromethorphan in total membrane homogenate from rat cerebellum (Tyndale et al., 1999), as shown through inhibition of dextromethorphan metabolism by two CYP2D-specific inhibitors. CYP2D18, an isoform identified in rat brain, but not in the liver, was also found to metabolize the CYP2D substrates imipramine (Kawashima et al., 1996) and desipramine (Thompson et al., 1998). This work demonstrated that CYPs expressed in brain tissue could metabolize their known xenobiotic substrates.

1.1.4 Role of the CYP2 subfamily in the brain

The CYP2 subfamily may be an important group of P450 enzymes in the brain. As described above, many isoforms belonging to the CYP2 family are responsible for metabolizing many CNS-acting drugs and are expressed widely throughout the brains of multiple species. Many CYP2 isoforms expressed in the brain can metabolize endogenous CNS substrates such as neurotransmitters and steroids, which suggests that brain expression of this CYP family might also play a role in endogenous neuronal processes. Furthermore, variation is observed in the enzymatic activity of CYP2 enzymes: the genes encoding CYP2 enzymes are genetically

variable and the activity of these enzymes can be induced or inhibited by xenobiotics (Zanger and Schwab, 2013). Variation in hepatic CYP activity can influence the subsequent response to their drug substrates (Zanger and Schwab, 2013), which suggests that brain CYP activity could also play a role in drug response if genetics and/or xenobiotic exposure alters the brain expression of CYP2 enzymes.

CYP2C protein expression has been characterized in rat brain and more recently in the human brain for the isoforms CYP2C9 and CYP2C19 (Booth Depaz et al., 2015). Little is known about whether these enzymes are active in the human brain. The only evidence of brain CYP2C activity is the metabolism of arachidonic acid to epoxyeicosatrienoic acid in rat astrocytes that express CYP2C11; however, it is possible that other CYP isoforms expressed in rat astrocytes with epoxygenase activity could contribute to this (Alkayed et al., 1996). Functional genetic polymorphisms exist for CYP2C9 (Aithal et al., 1999; Lee et al., 2002; Kosaki et al., 2004) and CYP2C19 (Hulot et al., 2006; Frere et al., 2009); therefore it is possible that variation in brain expression could influence the activity of these enzymes.

CYP2E1 can metabolize the muscle relaxant chlorzoxazone, anesthetics such as isoflurane, ethanol, and toxic industrial chemicals such as aniline and benzene (Gut et al., 1996). CYP2E1 from rat brain microsomes metabolizes chlorzoxazone (Upadhya et al., 2000), suggesting that brain CYP2E1 is metabolically active. CYP2E1 is induced in the liver by ethanol, as shown by findings in rats treated with ethanol and from human alcoholics, and this is believed to be one mechanism that contributes to ethanol tolerance (Lieber and DeCarli, 1968; Tsutsumi et al., 1989; Lieber, 1999; Zhukov and Ingelman-Sundberg, 1999). This induction has also been implicated in alcohol hepatotoxicity due to the ability of CYP2E1 to generate reactive oxygen species (Zhukov and Ingelman-Sundberg, 1999; Järveläinen et al., 2000). CYP2E1 expression is also induced by ethanol in rat brain (Roberts et al., 1994), and is higher in specific brain regions of human alcoholics (Howard et al., 2003; Toselli et al., 2015a). Nicotine is another drug that can induce brain CYP2E1 in rats (Howard et al., 2003; Joshi and Tyndale, 2006a) and monkeys (Joshi and Tyndale, 2006b; Ferguson et al., 2013), and higher expression in specific regions has been reported in post-mortem brains of smokers (Howard et al., 2003; Toselli et al., 2015a). Smoking and alcoholism have high co-morbidity and use of either cigarettes or alcohol is higher with co-use (Pomerleau et al., 1997; Bobo and Husten, 2000; Room, 2004); therefore, the effect of both ethanol and nicotine on brain CYP2E1 expression could have significant impact on its

activity. The combination of ethanol and nicotine treatment can increase CYP2E1 protein in frontal cortex and putamen in monkeys (Ferguson et al., 2013), suggesting that brain CYP2E1 activity could be higher following co-use. Given that CYP2E1 can generate reactive oxygen species and increases in CYP2E1 expression have been shown to correlate with damage to brain tissue in rats (Zhong et al., 2012), CYP2E1 in the brain may be an important contributor to the detrimental effects of chronic alcohol use and other drugs that may influence expression of this enzyme.

CYP2D from rat brain microsomes can metabolize its typical substrates (Tyndale et al., 1999), indicating that brain CYP2D can metabolize the same substrates as its hepatic counterpart. The *CYP2D6* gene is highly polymorphic, leading to variation in CYP2D6 levels among humans; this variation can influence enzyme function as *CYP2D6* genotype is associated with differences in metabolism of CYP2D substrates (Gaedigk, 2008). Given that many CNS-acting drugs are CYP2D substrates, variation in CYP2D activity in the brain may alter the effects of these drugs. The CYP2D substrate codeine, an analgesic prodrug, was used to prove that specifically altering CYP2D activity in the brain, but not in the liver, could influence drug response. Codeine is metabolized by CYP2D into morphine, which is the active metabolite with analgesic properties (Adler, 1955). Codeine readily crosses the blood-brain barrier due to its lipophilic nature, while morphine is actively transported out of the brain via transporters (Oldendorf, 1972; Bouw, 2000), which delays its entry into the brain and its analgesic action. Despite this delay in morphine entry, an analgesic response to codeine occurs within the first 30 minutes after codeine administration. This early analgesic response suggests that codeine, which can accumulate in the brain faster than morphine, could be converted to morphine in the brain. Introcerebroventricular (ICV) injection of chemical inhibitors selective for CYP2D were used to inhibit rat brain, but not liver, CYP2D activity and resulted in a reduction in the analgesic response to codeine given both subcutaneously (Zhou, 2013) and intraperitoneally (McMillan and Tyndale, 2015). This reduction in analgesia occurred within the first 30 minutes after both forms of codeine administration but was not found at later time points; these later time points would correspond to analgesia from morphine produced in the periphery by hepatic CYP2D which had then crossed into the brain. Brain morphine levels within the first 30 minutes were lower after ICV pre-treatment with CNS inhibitor compared to vehicle, while plasma morphine levels were unchanged, suggesting that central inhibitor treatment did not affect hepatic codeine metabolism

and that the inhibitor effect on analgesia was due to specifically blocking brain metabolism. The opposite manipulation of CYP2D activity, where CYP2D activity was increased, was also tested. Nicotine can induce CYP2D in rat brain and not liver (Yue, 2008); codeine-induced analgesia following nicotine pretreatment was greater compared to saline pretreatment, suggesting that increasing CYP2D expression can increase the conversion of codeine into morphine (McMillan and Tyndale, 2015). These recent findings provided evidence that brain CYP2D activity could influence the response to a centrally acting drug.

Like CYP2D6, the gene that encodes human CYP2B6 is highly polymorphic (Lang, 2001). The **6* genetic variant of *CYP2B6* is found in 15-60% of people depending on the ethnic population (Zanger and Klein, 2013) and results in a reduction of protein levels in the liver (Lang, 2001), suggesting that levels of CYP2B could also be variable in the brain among different populations. This isoform is also induced or inhibited by a variety of drugs and environmental compounds in the brain (Miksys, 2000; Lee et al., 2006a; Ferguson et al., 2013), and this could further increase the level of inter-individual variation in CYP2B activity. Thus, many enzymes belonging to the CYP2 family may have important roles in moderating the local levels of their xenobiotic and endogenous substrates and possibly influence drug response.

1.2 CYP2B

1.2.1 Isoforms expressed in the brain

CYP2B has widespread expression throughout the brain in multiple species. The functional CYP2B isoform in humans is CYP2B6 (Nelson et al., 2004), which has been detected in the brain (Gervot et al., 1999; Miksys, 2003). Monkey CYP2B6, previously known as CYP2B17 (Uno et al., 2011), shares 91% amino acid homology to humans and is also expressed throughout the brain in African green monkeys (Lee et al., 2006a). In rats, there are five isoforms, CYP2B1, CYP2B2, CYP2B3, CYP2B12 and CYP2B15. Two pseudogenes, CYP2B14P and CYP2B16P, also exist (Trottier et al., 1996). The most abundant isoforms, CYP2B1 and CYP2B2, share 97% amino acid homology (Suwa et al., 1985), and the mRNA and protein of both isoforms have been reported in rat brain (Anandatheerthavarada et al., 1990; Schilter and Omiecinski, 1993; Miksys, 2000; Rosenbrock et al., 2001a). CYP2B3 mRNA and protein are expressed in the liver (Desrochers et al., 1996) but have not been examined in the brain. CYP2B12 and CYP2B15 are

expressed in rat skin cells; both are capable of epoxygenase activity but little else is known about their function (Du et al., 2004). CYP2B1 is the rat ortholog to human CYP2B6: its gene shares 80% nucleotide sequence identity with *CYP2B6* (Lewis et al., 1999) and has been shown to metabolize the same substrates and probe drugs as CYP2B6 (Phillips et al., 1985; Nakayama et al., 1993; Parmar et al., 1998). Five CYP2B isoforms are expressed in the mouse: CYP2B9, CYP2B10, CYP2B13, CYP2B19, and CYP2B23; CYP2B13, CYP2B19, and CYP2B23 mRNA were detected in the brain, with very low levels detected for CYP2B13 and CYP2B19 (Renaud et al., 2011) while another study found CYP2B10 mRNA, the mouse ortholog to human CYP2B6 (Sueyoshi et al., 1999), expressed in the cortex and hippocampus (Stamou et al., 2014).

1.2.2 CYP2B substrates

CYP2B substrates are usually lipophilic, basic, and medium-sized molecules (Lewis et al., 2004). In a review by Wang and Tompkins (2008), the contribution of hepatic CYP2B6 to drug metabolism was estimated to cover between 3-12% of clinically used drugs despite CYP2B6 expression being relatively low in the liver (2-10% of total CYP content) compared to other isoforms, such as CYP2C (20% of CYP liver content and 12% contribution to metabolism of clinically used drugs). CYP2B6 metabolizes clinically important drugs, many of which act in the CNS. Drug substrates include a number of chemotherapeutics, anti-virals, anti-arrythmics, and anti-malarials (Table 1) (Turpeinen and Zanger, 2012). Substrates that act in the CNS include the anti-depressant bupropion (Hesse, 2000), and a number of anti-epileptics, anaesthetics, analgesics, stimulants, opioids, and benzodiazepines (Turpeinen and Zanger, 2012). A number of clinical drugs are predominantly metabolized by CYP2B6, such as bupropion, the HIV drug efavirenz (Ward et al., 2003), the anti-malarial artemisinin (Svensson and Ashton, 1999), the analgesic propofol (Court et al., 2001), and the synthetic opioid methadone (Totah et al., 2008). Many CYP2B6 substrates are also metabolized by other CYPs, such as CYP3A4, where CYP2B6 is a high affinity/low capacity enzyme and CYP3A4 is a low affinity/high capacity enzyme. One example of this would be ketamine N-demethylation by CYP2B6 and CYP3A4 where CYP2B6 has an affinity for ketamine 16 times higher than CYP3A4 but the maximum velocity of the enzyme is three times lower than CYP3A4 (Yanagihara et al., 2001). This suggests that CYP2B6 may contribute more to the metabolism of its substrates at low substrate concentrations.

CYP2B is not only involved in the metabolic inactivation of its substrates but is also capable of metabolically activating pro-drugs, pro-toxins and pro-carcinogens from environmental sources. One example of a CYP2B pro-drug substrate is the anti-cancer drug cyclophosphamide, which is metabolized by CYP2B into cytotoxic active metabolites (Chang et al., 1993). CYP2B can also metabolize the organic pesticide chlorpyrifos into an active neurotoxic metabolite (Tang, 2001), and transform the flame retardant 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) into several different toxic metabolites that can act as endocrine disruptors (Erratico et al., 2013; Feo et al., 2013). Pro-carcinogens found in tobacco smoke, specifically the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are also metabolically activated by CYP2B into metabolites that can form DNA adducts (Dicke et al., 2005).

In addition to drug metabolism, there are endogenous CYP2B substrates important for CNS function. These include the neurotransmitters serotonin (Fradette et al., 2004) and anandamide (Sridar et al., 2011), and the steroids testosterone (Imaoka et al., 1996), estrone and 17β-estradiol (Ohe et al., 2000). The contribution of CYP2B in regulating the levels of these endogenous molecules, in addition to those of its drug substrates, could have important consequences for CNS homeostasis.

Table 1. Examples of CYP2B substrates

Examples of non-CNS substrates		
	Drug	**Drug Class**
Exogenous	Artemisinin	Anti-malarial
	Cyclophosphamide	Chemotherapeutic
	Ifosfamide	Chemotherapeutic
	Tamoxifen	Chemotherapeutic
	Efavirenz	Anti-retroviral
	Nevirapine	Anti-retroviral
	Loperamide	Gastrointestinal
	Verapamil	Anti-arrhythmic
Examples of CNS substrates		
	Drug	**Drug Class**
Exogenous	Aminopyrine	Analgesic
	Methadone	Analgesic
	Meperidine	Analgesic
	Tramadol	Analgesic
	Propofol	Anaesthetic
	Ketamine	Anaesthetic
	Lidocaine	Anaesthetic
	Clobazam	Anxiolytic
	Diazepam	Anxiolytic
	Bupropion	Anti-depressant /Smoking cessation agent
	Fluoxetine	Anti-depressant
	Sertraline	Anti-depressant
	S-mephenytoin	Anti-convulsant
	S-mephobarbital	Anti-convulsant
	Nicotine	Stimulant
	N-ethyl-3,4-methylenedioxy-amphetamine (MDE)	Stimulant
	Nicotine-derived nitrosamine ketone (NNK)	Pro-carcinogen
	2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47)	Flame retardant
	Chlorpyrifos	
	N,N-Diethyl-meta-toluamide (DEET)	Pesticide Insect repellant
Endogenous substrates		
	Substrate	**Class**
	Testosterone	Steroid
	Estrone	Steroid
	Serotonin	Steroid
	17β-estradiol	Neurotransmitter
	Anandamide	Neurotransmitter

1.2.3 Sources of CYP2B variation

Early investigations of CYP2B expression in the liver observed large inter-individual variation (Code et al., 1997; Ekins et al., 1998; Gervot et al., 1999; Stresser and Kupfer, 1999), which contributes to the range of percent in CYP content attributed to CYP2B expression. This led to the determination of factors that cause CYP2B variation. As mentioned previously, the *CYP2B6* is highly polymorphic (Lang, 2001), and therefore a possible source of variation. *CYP2B6* expression can also be influenced by exposure to chemical compounds, such as phenobarbital, a known inducer of CYP2B and other CYP subfamilies (Chang et al., 1997). *CYP2B6* expression in monkeys, and Cyp2b1 and Cyp2b10 expression in the rat and mouse are also inducible by chemical compounds such as phenobarbital (Botelho et al., 1979; Thomas et al., 1981; Honkakoski and Lang, 1989; Schoedel et al., 2003); therefore exposure to exogenous compounds could also influence CYP2B expression in humans and other species. Furthermore, the influence of genetic variation and exposure to exogenous compounds on CYP2B expression could also occur in the brain since genetic variants are within the whole individual and exogenous compounds could penetrate the brain.

1.2.3.4 Genetic variation in *CYP2B6*

The influence of *CYP2B6* polymorphisms on drug response has been well characterized in the literature. Early studies mapping the *CYP2B6* gene indicated the existence of genetic variation: the gene was first identified in humans by Miles et al. (1988), where alternative splicing was found from two cDNA clones isolated from liver and Yamano et al. (1989) found polymorphic DNA fragments using BamHI and BglII restriction enzymes and cDNA human liver libraries. The first study to systematically examine *CYP2B6* polymorphisms found six alleles, labeled **2-7* in a Caucasian population (Lang, 2001). These alleles consisted of one or more single nucleotide polymorphisms in *CYP2B6* that resulted in amino acid changes in its protein product. Significantly lower protein expression was found in the livers that were positively genotyped for the C1459T mutation, which is present in both **5* and **7*. The livers that were positively genotyped for the **6* variant, which contains two mutations (G516T and A785G), also had lower protein expression compared to wildtype but this was not significantly different. It is possible that the sample size (three **6* homozygotes, ie. individuals who have two copies of **6*) was too small to detect significant differences, as later studies have confirmed that the **6* variant is

indeed associated with lower CYP2B6 protein levels in the liver (Xie et al., 2003; Hesse et al., 2004; Desta et al., 2007; Hofmann et al., 2008). The most frequent alleles in the Caucasian population analyzed by Lang et al. (2001) were as follows: the C1459T mutation (**5* and **7*) with a frequency of 14%, the A785G mutation (**4*; present in **7* and **6*) with a frequency of 33%; and G516T (present in **6* and **7*) with a frequency of 29%. Currently, there are 38 alleles reported for *CYP2B6* according to the *CYPalleles* website (http://www.cypalleles.ki.se/cyp2b6.htm, last accessed on August 20 2015). These alleles are also found in other ethnic populations, such as Hispanic-Americans, African-Americans and Asians (Lamba et al., 2003; Solus et al., 2004; Klein et al., 2005; Xu et al., 2007; Li et al., 2012).

The **6* variant is the most common functional allele, with an approximate frequency of 30% in Caucasians (Turpeinen and Zanger, 2012), 30-50% of African-Americans and 15-20% of Asians (Turpeinen and Zanger, 2012). This variant is associated with lower CYP2B6 protein in the liver compared to individuals wildtype for *CYP2B6*. The G516T mutation in **6* results in aberrant splicing of CYP2B6 pre-mRNA and thus reduces the levels of mRNA available for translation (Hofmann et al., 2008).

Lower protein due to less available mRNA has been shown to impact the activity of the enzyme. This was first shown in the study by Lang et al. (2001), where S-mephenytoin N-demethylation was measured in liver microsomes and livers genotyped positive for the **6* variant had approximately 50% of the activity found in livers from wild type individuals. Human liver microsomes homozygous for **6/*6* were found to have lower efavirenz hydroxylation compared to wildtype livers (Desta et al., 2007; Xu et al., 2012), consistent with the lower activity reported by Lang et al. (2001). *In vivo* data also suggested that the **6* variant reduces CYP2B6 catalytic activity: individuals homozygous for *6 had higher plasma efavirenz levels compared to wild type individuals during efavirenz treatment (Tsuchiya et al., 2004; Rotger et al., 2007). The effect of the variant on bupropion and efavirenz hydroxylation has also been assessed using recombinant expression systems in insect, bacterial, or primate cell lines (Ariyoshi et al., 2011; Zhang et al., 2011; Xu et al., 2012; Radloff et al., 2013); however, the **6* variant had higher affinity (K_m) and maximum velocity (V_{max}) compared wild type *CYP2B6* for bupropion and efavirenz hydroxylation. The **6* variant also appears to increase catalytic activity for cyclophosphamide hydroxylation in human liver microsomes (Xie et al., 2003) and for 7-

ethoxycoumarin demethylation in bacterial cells (Ariyoshi et al., 2001), which suggests that the effect of **6* on CYP2B6 activity may be substrate dependent.

The differences in *CYP2B6*6* activity could be due to the impact of the two single nucleotide polymorphisms (SNPs) found in **6*, G516T and A785G, where cyclophosphamide hydroxylation is increased, compared to wild type, when both SNPs are present (**6*), but decreased when only the A785G mutation is present (**4* variant) (Ariyoshi et al., 2011). Opposite effects of G516T and A785G were also seen with efavirenz hydroxylation, whereby lower activity was observed with **6* and higher activity was observed with **4* compared to wild type expressed in insect cells (Ariyoshi et al., 2011). Furthermore, lower protein expression with at least one copy of **6* but higher catalytic activity for nicotine, although not significant, was reported in human liver bank tissues (Al Koudsi and Tyndale, 2010). Together these data suggest that lower protein expression due to the **6* variant could result in lower activity, but either of the two SNPs present in **6* could either enhance or reduce the enzyme's catalytic activity in a substrate-dependent manner.

The effect of *CYP2B6* genetic variation on CYP2B6 activity has primarily been investigated in the liver; however, this type of genetic variation is expected to have a functional impact in all tissues where the protein is expressed, including the brain. One study from our group examined CYP2B6 protein expression in genotyped post-mortem human brain (Miksys, 2003). Brain from individuals genotyped as either homozygous or heterozygous for the C1459T mutation, classified as the **5* allele and also found in **7*, had lower CYP2B6 protein compared to brain from wild type subjects. This suggested that genetic variation could influence CYP2B6 protein levels in the human brain, although the sample size used in the study was small for investigating genotype effects (N = 26) and alteration of CYP2B6 expression by potential pre-mortem exposure to inducers or inhibitors could not be excluded.

1.2.3.5 Non-genetic variation in CYP2B expression

Part of the variability in CYP2B expression is due to the influence of exogenous compounds. Many commonly prescribed drugs, environmental chemicals and toxins, steroid hormones, and compounds found in food are inducers or inhibitors of CYP2B in humans or other species, such monkey, rat, and mouse (Martignoni et al., 2006). Induction or inhibition of CYP2B can be organ-specific, whereby an inducer can increase protein levels in either liver or brain, or in both tissues (Gervot et al., 1999; Miksys et al., 2000a; Schilter et al., 2000; Schoedel et al., 2001).

Furthermore, many CYP2B inducers or inhibitors are substrates for the enzyme, thereby influencing their own metabolism with subsequent re-exposure (Chang et al., 1997).

1.2.3.5.1 CYP2B inducers

Phenobarbital (PB) is a typical CYP2B inducer. Rat CYP2B was first identified as a phenobarbital-inducible CYP in studies where CYP2B protein was higher in liver from rats treated with PB (Botelho et al., 1979; Guengerich et al., 1982). PB treatment can also induce CYP2B protein in human cultured hepatocytes (Chang et al., 1997), in mouse liver (Honkakoski and Lang, 1989) and in monkey liver (Schoedel et al., 2003; Lee et al., 2006b). The mechanism underlying this induction in the liver involves activation of the constitutive androstane receptor (CAR), which binds to the phenobarbital response element (PBREM) on the *CYP2B* gene to elicit transcription (Honkakoski et al., 1998; Sueyoshi et al., 1999). The anti-epileptic drug phenytoin is another typical CYP2B inducer in human hepatocytes (Wang et al., 2004) and rat liver (Diwan et al., 1988), also via CAR (Wang et al., 2004). The classic CYP3A4 inducer rifampicin can also increase CYP2B protein in human primary hepatocytes (Chang et al., 1997), by activating the pregnane X receptor (PXR) which also binds to the PBREM element on the *CYP2B* gene (Goodwin et al., 2001). In addition, ethanol, a commonly used drug, can induce CYP2B in rat (Schoedel et al., 2001) and monkey liver (Ferguson et al., 2012; Ferguson et al., 2013). The mechanisms underlying induction are thought to be both transcriptional and non-transcriptional in rat (Schoedel et al., 2001) but non-transcriptional in monkey (Ferguson et al., 2012). CYP2B substrates that can induce CYP2B in the liver include methadone (Campbell et al., 2013), cyclophosphamide (Graham et al., 1983; Martin et al., 2003), and the pesticide endosulfan (Casabar et al., 2010). These chemicals can also induce CYP2B protein levels in the brain. PB can induce CYP2B protein in the brain of rats (Schilter and Omiecinski, 1993; Schilter et al., 2000) and monkeys (Lee et al., 2006b). Phenytoin can also induce CYP2B in the rat brain (Kempermann et al., 1994; Rosenbrock et al., 1999). The mechanisms underlying regulation of *CYP2B* in the brain may be different from those in the liver, as nicotine can induce CYP2B in the brain but not the liver of rats (Miksys, 2000) and monkeys (Lee et al., 2006a).

Diet can also affect CYP2B expression. Vitamin D has been shown to induce CYP2B in human hepatocytes through binding of PXR and CAR (Drocourt et al., 2002). CYP2B in mouse liver can also be induced by fasting through CAR activation (Ding et al., 2006). There is evidence that

glucocorticoids can also induce CYP2B expression in the liver; however, this has only been shown in rodents treated with dexamethasone (Schuetz et al., 1984; Corcos, 1992). Dexamethasone does not induce CYP2B expression in human hepatocytes but can potentiate its induction by PB through up-regulation of CAR mRNA (Pascussi et al., 2000). This suggests that glucocorticoids can influence CYP2B expression in multiple species, although this may occur through different mechanisms, for example by direct activation of CAR in rodents, and by indirect induction of the amount of CAR available for activation in humans.

1.2.3.5.2 CYP2B inhibitors

Many drugs, environmental toxins, and dietary sources are also CYP2B inhibitors, which could lead to clinical consequences involving drug-drug interactions. Some of these drugs are CYP2B substrates (Walsky et al., 2006), which can inhibit the enzyme by virtue of occupying the catalytic site and competing with other substrates (Reed et al., 2010) or through conversion to a reactive intermediate that binds to the enzyme (mechanism-based inhibition [MBI]) (Copp, 2003). The antiretroviral drugs ritonavir, efavirenz, and nelfinavir can inhibit CYP2B6 *in vitro* (Hesse et al., 2001). Thirty compounds out of over 100 tested *in vitro* could inhibit CYP2B6 activity by over 50% (Walsky et al., 2006). Clopidogrel and ticlopidine, two platelet aggregators, are potent MBI's of CYP2B6 (Richter et al., 2004). The anti-fungals clotrimazole and itraconazole, the anti-depressant sertraline, and the estrogen receptor modulator raloxifene are potent CYP2B6 inhibitors (Walsky et al., 2006). Other anti-fungals such as voriconazole (Jeong et al., 2009), miconazole, and sulconazole (Zhang et al., 2002), anti-depressants such as paroxetine, norfluoxetine, and fluvoxamine (Walsky et al., 2006), and the Parkinson's disease drug selegiline (Sridar et al., 2012) also inhibit CYP2B activity. Organophosphate pesticides such as chlorpyrifos and fenitrothion can inhibit CYP2B in human liver microsomes (Abass et al., 2009; Abass and Pelkonen, 2013). Dietary inhibitors include curcumin, a component of the spice turmeric (Appiah-Opong et al., 2007), ε-viniferin, the dimer of resveratrol found in wine (Piver et al., 2003), and extracts from gingko biloba herb (Lau and Chang, 2009). Furanocoumarins, compounds present in fruit such as grapefruit and other citrus varieties, can inhibit many CYPs including CYP3A4 and CYP2D6 (Guo and Yamazoe, 2004). The furanocoumarin 8-methoxypsoralen (8-MOP) is an MBI for CYP2B1 in rat and CYP2A6 in humans (Koenigs et al., 1997; Koenigs and Trager, 1998).

While these compounds can inhibit CYP2B6 activity, some of them can also inhibit other CYPs with similar potency (Turpeinen and Zanger, 2012), which makes their use difficult for studying selective metabolism by CYP2B6. However, a few selective inhibitors for CYP2B are available. The anti-cancer agent thioTEPA can selectively inhibit CYP2B6 *in vitro* by working as an MBI and can reduce plasma levels of the metabolite of CYP2B substrate cyclophosphamide *in vivo*. Another selective MBI of CYP2B1 is C8-xanthate (C8X), a derivative of alkyl xanthates (Yanev, 1999; Yanev et al., 2000).

1.2.4 Functional impact of variation in CYP2B activity

The effect of altered CYP2B activity on drug response has been exemplified using efavirenz and bupropion. *CYP2B6* genetic variation, most commonly studied with the **6* variant, can influence *in vitro* efavirenz metabolism and *in vivo* plasma efavirenz concentrations (Tsuchiya et al., 2004; Desta et al., 2007). This has also been shown for other *CYP2B6* gene variants (Holzinger et al., 2012; Li et al., 2012; Colic et al., 2014): other alleles that carry the G516T variant found in **6* (ie. **20* and **27*), T983C (found in **16* and **18* alleles), C1459T (found in **5* and **7*), and an intron variant (rs4803419) were found to decrease efavirenz metabolism. Individuals positively genotyped for the loss-of-function variants had higher efavirenz concentrations and experienced more of the CNS side effects associated with efavirenz treatment (Haas et al., 2004; Gounden et al., 2010). Thus, variation in CYP2B6 activity is an important factor in determining efavirenz treatment doses that minimize side effects while maintaining the clinical effect.

The effect of the anti-depressant and smoking cessation agent bupropion can also be influenced by *CYP2B6* variation. CYP2B6 is the main enzyme responsible for metabolizing bupropion to its active metabolite hydroxybupropion (Hesse, 2000). Genetic variation in *CYP2B6* can alter bupropion metabolism *in vitro*, where the **6* variant showed decreased catalytic efficiency for bupropion along with **4* to**9* variants when compared to wild type in recombinant systems (Zhang et al., 2011). *In vivo*, *CYP2B6*6* was associated with lower hydroxybupropion levels in serum at steady state (Hoiseth et al., 2015), and **6* and **18* were associated with lower hydroxybupropion levels in plasma and urine at steady state (Benowitz et al., 2013). Genetic variation in *CYP2B6* was a significant factor in the variability of hydroxybupropion plasma levels in one clinical smoking cessation trial, where this variation was further associated with smoking cessation outcome (Zhu et al., 2012). These results suggest that the hydroxyl metabolite

is the active compound mediating bupropion's effect on smoking cessation and that differences in CYP2B6 activity between people could influence this effect.

These two examples demonstrated that systemic levels of these CYP2B substrates can be altered by variation in CYP2B activity. Genetic variation in *CYP2B* and exposure to environmental inducers or inhibitors could also alter the levels and/or activity of CYP2B in the brain; therefore it is possible that altered brain CYP2B activity could also influence the brain levels of its substrates. Both efavirenz and bupropion can enter the brain (Suckow et al., 1986; Tashima et al., 1999), with the latter believed to exert its therapeutic effects within the brain (Nomikos et al., 1992), suggesting the possibility that altered CYP2B activity in both liver and brain could be influencing brain substrate levels. Chemical inducers, such as nicotine, can increase CYP2B protein in the brain but not the liver (Miksys, 2000; Lee et al., 2006a; Ferguson et al., 2013), suggesting that brain CYP2B activity can be altered independently of hepatic activity.

1.2.5 Functional impact of CYP2B variation within the brain

While plasma levels of the substrates efavirenz and bupropion were affected by CYP2B variation, brain levels of other CYP2B substrates may also be affected by CYP2B variation and thus alter drug response. The anaesthetic propofol, which acts on gamma-aminobutyric acid (GABA) receptors in the CNS (Altomare et al., 2003), is metabolically inactivated by CYP2B6 (Oda et al., 2001). Brain propofol levels predicted the anaesthetic response (measured as sleep time) better than plasma propofol levels in patients (Liu, 2009), indicating that brain levels of the drug were more likely to mediate its effects. Also, smokers were found to require higher doses of propofol to elicit a hypnotic effect (Lysakowski et al., 2006), which suggests that their brain propofol levels might be lower at a given dose compared to non-smokers. This would be consistent with the higher protein expression of CYP2B observed in the post-mortem brains of human smokers (Miksys, 2003).

Another CNS-acting CYP2B substrate for which variation in brain CYP2B activity may have important consequences is the pesticide chlorpyrifos. Chlorpyrifos-oxon, formed by CYP2B (Tang, 2001), inhibits acetylcholinestase activity (Sultatos, 1994), causing cholinergic overstimulation that results in neurotoxicity and cognitive deficits (Steenland, 2000). Chlorpyrifos-oxon-mediated neurotoxicity was thought to originate in the brain rather than the liver, due to the rapid metabolism of the oxon by esterases in the blood (Costa, 1990; Drevenkar,

1993). This is supported by evidence that chlorpyrifos can be metabolized to the chlorpyrifos-oxon in whole brain homogenates from rat (Chambers, 1989).

1.2.6 Rat model of CYP2B variation in the brain

Investigating the role of variation in brain CYP2B activity is a challenge in humans. Brain drug levels cannot be directly measured in humans. The only technique available to assess this is through positron emission tomography (PET) imaging, which measures the accumulation of radio-labeled drug (Fowler et al., 1999). However, there are limitations to this method in studying variation in enzyme activity, as the radio-label may be positioned on the drug metabolite(s) and therefore parent and metabolite levels would be hard to distinguish. Variation in CYP2B activity could also occur in the liver where the hepatic form of the enzyme is expressed, and the contribution of this variation to systemic levels might influence the levels of drug that reach the brain. Therefore, while it may be possible to look at the contribution of CYP2B genetic variation in the brain in humans through imaging studies, these studies would require radiolabel design to ensure that radioactivity measures the parent drug. PET imaging studies are also more costly, thus, an animal model could first be used to investigate whether there is an effect of altering CYP2B activity in the brain without altering activity in the liver.

The rat is a suitable animal in drug metabolism models because the CYP2B homolog to human CYP2B6, CYP2B1, is expressed in the brain (Schilter and Omiecinski, 1993; Miksys, 2000). CYP2B1 metabolizes the same substrates as CYP2B6, including propofol (Yamazaki et al., 2006) and nicotine (Nakayama et al., 1993), and behavioural effects of these substrates measured in the rat are similar to that in humans (Cavazzuti et al., 1991; Rose and Corrigall, 1997). Finally, rat CYP2B can be inhibited by the same selective MBI, C8X, that inhibits CYP2B6, (Yanev, 1999) and can be induced in the brain by nicotine (Miksys, 2000), indicating that CYP2B activity in the liver and brain can be manipulated by pharmacological methods in this species.

1.2.6.1 Brain CYP2B inhibition using MBIs

Two MBIs that have been used to inhibit *in vivo* CYP2B activity in the brain are C8X and 8-MOP. C8X was first characterized as an MBI *in vitro* by Yanev et al. (1999), where C8X and a variety of alkyl xanthates were tested for their ability to inhibit multiple CYPs (Yanev, 1999). C8X inhibited CYP2B1 with an inactivation potency (K_i) of 2.4 µM. CYP2E1 was inhibited by

C8X at a much lower potency (K_i = 60 µM), which indicates that selective inhibition of CYP2B can occur at low concentrations of C8X. The inhibition of CYP2B1 by C8X was shown to be mechanism-based, whereby C8X was later determined to inactivate CYP2B1 by reducing its ability to receive electrons from the NADPH oxidoreductase (Yanev et al., 2000). The MBI 8-MOP, also a potent inhibitor of CYP2A6 in humans, inhibits CYP2B1 *in vitro* by binding to the CYP2B apoprotein when converted to its reactive intermediate (Koenigs and Trager, 1998). Using reconstituted CYP2B1, 8-MOP had a K_i of 2.9 µM with the potencies of other furanocoumarins ranging from 0.3-1 mM.

MBIs were recently used to test rat CYP2B brain activity *in vivo* (Miksys and Tyndale, 2009). Radiolabeled 8-MOP (^{3}H-8-MOP) was injected intracerebrally (IC) into the left frontal cortex. ^{3}H-8-MOP is metabolized by CYP2B to ^{3}H-dihydro diol, the reactive intermediate that covalently binds to CYP2B (Koenigs and Trager, 1998). Therefore, if CYP2B was active the reactive intermediate would be formed and bind to the enzyme. CYP2B was immunoprecipitated from the brains of MBI-treated rats, where increasing radiolabeled protein was measured with increasing CYP2B-specific antibody, indicating that the radiolabeled intermediate was bound to CYP2B. Then, to test the specificity of 8-MOP metabolism by CYP2B, C8X, another MBI structurally distinct from 8-MOP, was injected into the right frontal cortex before ^{3}H-8-MOP administration (Miksys and Tyndale, 2009). Frontal cortex tissue pre-treated with C8X did not show any increase in radiolabeled protein immunoprecipitated with the CYP2B-specific antibody, suggesting that C8X inactivated CYP2B and prevented the formation and subsequent binding of the radiolabeled intermediate to the enzyme.

CYP2B activity can also be inhibited in the whole brain by MBI injection into the lateral ventricles (Khokhar, 2011). ICV injection is a method to obtain whole brain drug exposure, as cerebrospinal fluid in the lateral ventricles flows throughout the brain before leaving through the sub-arachnoid space (Abbott, 2004). Radioactivity was measured in CYP2B-immunoprecipitated brain stem and frontal cortex tissue from rats treated with ICV ^{3}H-8-MOP, indicating that CYP2B was able to metabolize the radioactive MBI to the ^{3}H-dihydro diol, which could in turn bind to the enzyme. Pre-treatment with ICV C8X prior to ICV ^{3}H-8-MOP resulted in lower bound radioactivity compared to ICV pretreatment with C8X's vehicle in the same brain regions, demonstrating that MBI inhibition was selective for CYP2B. These experiments provided evidence that CYP2B is active in more than one brain region and that MBIs given ICV could

circulate throughout the brain and inactivate CYP2B, establishing the utility of MBIs in investigating brain CYP2B activity.

Using MBI's to inhibit CYP2B in the brain has advantages over competitive inhibitors. One advantage is that MBIs need to be bio-transformed by the enzyme in order to inhibit it, where their mechanism of action involves the metabolism of the MBI into a reactive intermediate that covalently binds to the enzyme, thus inactivating it. This provides a level of selectivity as MBIs need to interact with an active form of the enzyme to inhibit it. Another advantage of MBIs is in reducing ICV injection-related stress, as MBIs permanently inactivate the enzyme and can thus be injected several hours, or the day, prior to drug treatment. ICV injections can increase plasma corticosterone levels in mouse, suggesting that these injections can cause stress, which can in turn increase responding for drugs of abuse in self-administration paradigms (Kim et al., 1998; Sinha, 2008). Furthermore, many MBIs have clinical effects unrelated to their action as enzyme inhibitors; for example, the CYP2B MBIs clopidogrel and ticlopidine antagonize adenosine diphosphate receptors to prevent blood platelet aggregation (Richter et al., 2004). Although these actions in particular are not likely to influence CNS-mediated behaviour, longer times between MBI pretreatment and drug treatment reduce the potential influences of non-specific effects on the response to the test drug.

1.2.6.2 Brain CYP2B induction using nicotine

While inhibition of CYP2B activity is one way CYP2B source of variation in the brain, increased CYP2B activity could occur by exposure to chemical inducers or genetic variation that results in greater activity. Many of the chemical inducers described previously also induce CYP2B activity in the liver, which makes the contribution of brain induction to drug response difficult to determine. Nicotine, however, is capable of inducing CYP2B in the brain but not the liver, which provides a method to increase CYP2B activity selectively in the brain. Following chronic nicotine exposure in rats (Miksys, 2000; Khokhar, 2010) and monkeys (Lee et al., 2006a), CYP2B levels were higher in brain, but not in liver, consistent with the higher CYP2B6 levels observed in post-mortem brains of human smokers (Miksys, 2003).

The paradigm for chronic nicotine exposure in rats involves seven consecutive daily injections of nicotine at 1 mg/kg administered subcutaneously (SC). Higher CYP2B protein and mRNA levels were reported 4 hrs after the last nicotine injection in the brain stem, olfactory bulb, olfactory

tubercle, striatum and frontal cortex in rats (Miksys, 2000). Immunocytochemical staining displayed cell-specific increases in CYP2B-immunoreactivity following nicotine treatment, where induction increased CYP2B mainly in neurons. Time-course experiments showed maximal induction between 2-8 hrs after nicotine injection in the brain stem and after 8 hrs in the frontal cortex (Khokhar, 2010). Induction was still evident 24 hrs after the last nicotine injection in both regions and returned to baseline levels after 7 days. The increase in CYP2B brain levels seen after a 7-day nicotine treatment in Khokhar et al. (2010) was consistent with that reported in Miksys et al. (2000), where significant induction was found in the brain stem and frontal cortex but not in the cerebellum of rat. In both studies, liver CYP2B protein was not affected by the 7-day nicotine treatment, demonstrating that this paradigm increased brain but not hepatic CYP2B.

Chronic nicotine can also induce brain CYP2B in monkeys (Lee et al., 2006a). A different pattern of nicotine exposure was used in this species, with two days of two 0.05 mg/kg/SC injections, two days of 0.15 mg/kg/SC injections, and then 18 days of 0.6 mg/kg/SC, to give animals a daily dose similar to the average daily nicotine dose obtained by smokers (0.54 mg/kg) (Benowitz and Jacob, 1984). The 0.6 mg/kg dose was given in two 0.3 mg/kg/SC injections to obtain similar plasma nicotine levels (> 20 ng/ml) to those seen in smokers (19 to 50 ng/ml) (Lee et al., 2006a). With this nicotine treatment, CYP2B6 protein levels were higher in the frontal cortex compared to saline. There was no effect of nicotine treatment on CYP2B6 protein levels in the liver (Lee et al., 2006a), consistent with what was seen in rat. These studies show that nicotine can induce CYP2B specifically in the brain in multiple species, suggesting that nicotine could contribute to brain CYP2B variation in humans, and provides a paradigm to test the effect of brain CY2B induction in animal models.

1.2.6.3 Drug response in rat model of brain CYP2B variation

Brain CYP2B variation using the rat model was first characterized by previous work from our group, whereby MBI inhibition was used to reduce CYP2B activity and/or 7-day nicotine treatment was used to increase CYP2B activity (Khokhar, 2011; Khokhar, 2012). Brain drug levels and drug response to CYP2B substrates were measured after reducing or increasing CYP2B activity to demonstrate the functional impact of altered CYP2B activity in the brain. Using the two structurally different MBIs, C8X and 8-MOP, brain CYP2B activity was inhibited without altering liver CYP2B in rats and propofol drug response was tested (Khokhar, 2011).

Rats that were given ICV injections of either MBI 24 hours prior to propofol administration had increased brain propofol levels compared to their respective vehicle. ICV injections of either MBI did not alter plasma propofol levels nor did they alter hepatic CYP2B activity *ex vivo*, which demonstrates that the effect of ICV MBI treatment was due to CYP2B inhibition selectively in the brain. This inhibition was sufficient to alter propofol's behavioural response as the anaesthetic effect of propofol, sleep time, increased with ICV MBI treatment compared to ICV vehicle. Furthermore, brain but not plasma propofol levels were elevated by CNS inhibitor injection, and correlated with sleep time, confirming that brain propofol levels were associated with the behaviour. Brain CYP2B induction, using nicotine (Miksys, 2000; Khokhar, 2010), resulted in the opposite effect: CYP2B induction decreased brain propofol levels and decreased sleep time (Khokhar, 2011). These experiments were the first to prove that CYP2B activity in the brain could influence brain drug levels to an extent sufficient to alter drug response.

The same paradigm was also used to demonstrate that chlorpyrifos neurotoxicity was due to activation of chlorpyrifos to its metabolite chlorpyrifos-oxon in the brain (Khokhar, 2012). Inhibiting CYP2B selectively in the brain using the CYP2B MBIs was able to address this hypothesis. ICV pre-treatment by either C8X or 8-MOP increased chlorpyrifos levels and reduced chlorpyrifos-oxon levels in the brain but not in the liver. This suggests that inhibiting CYP2B in the brain reduced the formation of the chlorpyrifos-oxon. Furthermore, ICV MBI treatment reduced the inhibitory effect of the chlorpyrifos-oxon on brain cholinesterase activity. The reduction in metabolite levels after ICV MBI treatment was accompanied by reductions in the neurotoxic effects, which consist of hypothermia and behavioural impairments such as a reduced gait and loss of aerial righting reflexes. These experiments support the possibility that local activation of chlorpyrifos to the chlorpyrifos-oxon occurs in the brain. Administering the CYP2B MBIs after chlorpyrifos exposure as opposed to the pre-treatment paradigm in Khokhar (2012) was also able to reduce the chlorpyrifos-oxon behavioural impairments (Khokhar, 2014), suggesting that MBI treatment after chlorpyrifos exposure reduced the amount of chlorpyrifos-oxon in the brain by blocking subsequent formation of the metabolite. Reversal of chlorpyrifos neurotoxicity by MBI treatment not only supports a potential role for brain CYP2B activity in mediating its toxic effects, but also provides a target for treatment development. These experiments manipulating propofol and chlorpyrifos metabolism in the brain established that

brain CYP2B activity, independent of the liver, could influence brain drug levels and drug response.

1.2.7 Role of brain CYP2B in smoking behaviour

It is possible that brain CYP2B may play a role in nicotine-mediated behaviours such as smoking since nicotine is a substrate for CYP2B (Yamazaki, 1999). Smoking cessation studies suggest that *CYP2B6* genetic variation, specifically the **5 or *6* variant, can influence cessation outcomes (Lerman et al., 2002; Lee et al., 2007a). These alleles have previously been characterized to reduce CYP2B6 protein levels in the liver and are thus considered variants that reduce CYP2B6 metabolism (Lang, 2001; Lamba et al., 2003; Hofmann et al., 2008). Individuals who are genotyped positive for reduced-function alleles are identified as *CYP2B6* slow metabolizers. In the placebo group of a bupropion smoking cessation trial (Lee et al., 2007a), the percentage of smokers genotyped positive for the **6* allele that remained abstinent at the end of treatment and at 6-month follow-up was lower than smokers that were genotyped positive for the wild type allele. Another investigation of *CYP2B6* slow metabolizers in this bupropion smoking cessation trial genotyped smokers for the **5* allele and found smokers that were genotyped positive for this allele had a lower probability of being abstinent at the end of treatment (Lerman et al., 2002). Other behavioural measures assessed during cessation were different between genetic groups: time to relapse after the quit date was shorter and craving scores were higher for *CYP2B6*5* individuals compared to wild type (Lerman et al., 2002).

Despite these observations in abstinence after cessation, *CYP2B6* slow metabolizers do not have higher plasma nicotine levels when exposed to the same amount of nicotine (Lee et al., 2007b), which suggests that the influence of *CYP2B6* genetic variation in smoking could be due to lower expression and/or activity in the brain. In humans CYP2B6 can metabolize nicotine to a lesser extent compared to its main metabolizing enzyme CYP2A6 (Yamazaki, 1999), therefore extra-hepatic expression in the brain, where CYP2B concentrations have been reported as high or higher than in the liver in specific cell populations (Miksys, 2003), could contribute to nicotine drug response. Lower brain CYP2B6 protein levels were reported in brains genotyped for *CYP2B6*5* and higher levels were reported in the post-mortem brains of smokers (Miksys, 2003), which suggests that variation in CYP2B6 expression could possibly occur in the human brain.

The rat model of altered CYP2B activity in the brain, which changed brain levels of, and drug response to, propofol and chlorpyrifos, would determine whether brain CYP2B variation can influence nicotine levels in the brain and nicotine-mediated behaviours. CYP2B metabolizes nicotine into its inactive metabolite cotinine (Hammond et al., 1991). As CYP2B inhibition by MBIs was able to show increased propofol levels in the brain, increased nicotine levels would be expected in the brain after nicotine administration and possibly increase nicotine-mediated behaviour. Conversely, CYP2B induction by nicotine would be expected to reduce nicotine levels in the brain.

2 Nicotine and Smoking

Nicotine dependence from cigarette smoking is a worldwide problem, with almost a billion adults reported to be daily smokers in 2012 in a study that examined smoking prevalence in almost 200 countries (Ng et al., 2014). In Canada, 16% of people over 15 years old were reported current smokers in the 2012 Canadian Tobacco Use Monitoring Survery (CTUMS), which is lower than the reported 25% of current smokers from the 1999 CTUM survey (CTUMS, 2012) but still represents a substantial proportion of the population. While the percentage of smokers has decreased in Canada and other developed countries it continues to remain a major health problem in these countries, and the percentage of smokers is still high in developing countries. The number of daily smokers reported in the 2012 worldwide report (almost 1 billion) increased from that reported in 1980 (approximately 700 million) (Ng et al., 2014), indicating that smoking continues to be an important health issue globally. According to the World Health Organization, tobacco is still one of leading causes of preventable death and almost 6 million people die every year from tobacco-related illness (WHO, 2012). Therefore, strategies to reduce smoking populations are still important to develop.

2.1 Nicotine in cigarettes

Nicotine is an alkaloid naturally found in the leaves of the tobacco plant (Soloway, 1976). It is the primary psychoactive ingredient in cigarettes that contributes to smoking behaviour (Schmeltz and Hoffmann, 1977; Benowitz et al., 1983). Commercial cigarettes contain 10-14 mg

of nicotine and around 1-1.5 mg of this is absorbed by the body after smoking (Benowitz and Jacob, 1984). The pK_a of nicotine is 8.0, making it a weak base (Fowler, 1954). A large proportion of nicotine is un-ionized in cigarette smoke, due to a reported pH of 6.2 or higher (Brunnemann and Hoffmann, 1974), which allows for the rapid absorption of nicotine into the lungs. Nicotine is quickly absorbed into the blood stream from the lungs and enters the brain within 10-20 seconds after a single puff of a cigarette (Benowitz, 1990b; Benowitz, 1996; Rose et al., 2010), where it acts on central nicotinic acetylcholine receptors that activate the dopamine reward system. Nicotine's fast delivery and action in the brain are thought to play principal roles in the mechanisms underlying the development of tobacco dependence (Henningfield and Keenan, 1993; Kenny and Markou, 2006).

2.2 Pharmacological action of nicotine in the brain

2.2.1 Nicotinic acetylcholine receptors

Nicotine is a ligand for nicotinic acetylcholine receptors (nAChRs) which are expressed throughout the CNS (Sargent, 1993). Its actions at these receptors in the brain are considered the mechanism(s) behind the reinforcing effects of nicotine (Picciotto et al., 2008). The nAChRs are cationic ligand gated channels that are created by various configurations of five subunits. These subunits are separated into two subfamilies, α and β, of which nAChRs can form homomeric or heteromeric pentamers. There are 12 subunits expressed in the brain: α2-α10, and β2-β4 (Sargent, 1993; Gotti and Clementi, 2004). These receptors can be formed by α and β subunits to form heteromers, usually in a 2:3 stoichiometry of α and β subunits, or five α subunits to form homomers. Two subunits form a ligand binding site, therefore the number of ligand binding sites is dependent on the pentamer configuration. Ligand binding sites can be formed by α2-α4 and α6 subunits and β2 and β4 in heteromeric pentamers with the exception of α5 and β3, as neither subunit contains the complementary binding component to create the binding site; however, all subunits can form ligand binding sites with their neighbouring subunits in homomeric pentamers, which can be formed by α7-α9 subunits (Sine, 2002).

The α4β2, α5-, α6-, and α7-containing receptors in the brain are suggested to be involved in nicotine dependence (Paterson, 2009). These receptors are expressed in many regions of the brain, but their expression in the mesolimbic dopamine system (Klink et al., 2001) is important

for nicotine dependence, as activation of this system is thought to mediate reward (Yokel and Wise, 1975). The α4β2 receptor was first implicated in nicotine dependence because they account for the largest percentage (90%) of high-affinity binding sites for nicotine in the brain (Whiting and Lindstrom, 1988), and chronic nicotine can up-regulate these subunits (Marks et al., 1983; Benwell et al., 1988; Breese et al., 1997; Buisson and Bertrand, 2001). Furthermore, α4 knockout mice show greater nicotine reward in a conditioned place preference model (Tapper et al., 2004), while β2 knockout mice do not self-administer nicotine (Picciotto et al., 1998). Nicotine-mediated dopamine release in the ventral striatum, which is a component of the mesolimbic dopamine system, is impaired in β2 knockout mice, suggesting that this subunit may be required for nicotine reinforcement (Picciotto et al., 1998). Furthermore, partial agonists for the α4β2 receptor, such as sazetidine-A, or varenicline, can reduce nicotine self-administration (Levin et al., 2010; O'Connor et al., 2010).

The α5 subunit is expressed in similar regions as the α4 and β2 subunits, such as the cortex, hippocampus, striatum, and mesolimbic regions, where it can form a heteromeric receptor with both subunits (Gotti and Clementi, 2004). This subunit may be involved in regulating nicotine intake as α5 knockout mice displayed increased nicotine intake during nicotine self-administration, which was reversed with re-expression of α5 in the medial habenula (Fowler et al., 2011).

The α7 subunit is expressed in the cortex, hippocampus, amygdala and mesolimbic regions (Feduccia et al., 2012). Receptors containing the α7 subunit can be found on pre-synaptic glutamatergic synapses, including glutamate afferents to the brain reward system, which could play a role in nicotine reinforcement (Jones and Wonnacott, 2004). Administering methyllycaconitine, a selective antagonist for this subunit, reduced intracranial self-stimulation (Panagis et al., 2000) and reduced nicotine self-administration in rats (Markou and Paterson, 2001). Consistent with this, α7 knockout mice show reduced nicotine self-administration behaviour compared to wild type mice (Besson et al., 2012), suggesting that this subunit is involved in the rewarding effects of nicotine.

The α6 subunit is expressed in dopaminergic neurons (Le Novere et al., 1996). Similar to α7, administering the α6 selective alpha-conotoxin H9A;L15A can block nicotine's reinforcing effect as measured by conditioned place preference and withdrawal-associated conditioned place

aversion in mice (Jackson et al., 2009). Knockout mice also show a rightward shift in the dose-response curve for nicotine conditioned place preference compared to wild type mice (Sanjakdar et al., 2015), which suggests that α6-containing receptors may also contribute to nicotine reinforcement. Together, this evidence from rodent models suggests that nicotine's effects in the brain are mediated by multiple nAChR subtypes.

2.2.2 Brain reward system

The nAChRs associated with nicotine dependence are expressed in brain regions that comprise the brain reward system. This system consists of dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens and prefrontal cortex (Swanson, 1982). The role of dopamine in reward behaviour was first established in animal studies using intracranial brain self-stimulation, where dopamine antagonists reduce this behaviour (Rolls et al., 1974; Yokel and Wise, 1975). These pathways are thought to mediate the reinforcing effects of naturally rewarding stimuli such as food or sex (Wise et al., 1978a; Wise et al., 1978b), where feelings of pleasure and hedonia following these rewards motivate the continuation of this behaviour. This is important for natural rewards because they are essential to the survival of the organism and its species; however, drugs of abuse, including nicotine, can also activate this system (Di Chiara and Imperato, 1988) which leads to the development of drug-seeking behaviour.

Nicotine activates nAChRs in the VTA (Pidoplichko et al., 1997), resulting in dopamine release in both the nucleus accumbens (Nisell et al., 1994) and prefrontal cortex (Drew et al., 2000). Injecting the nAChR antagonist, dihydro-β-erythroidine (DHβE), into the VTA decreases nicotine self-administration in rats (Corrigall et al., 1994), suggesting that dopamine release from the VTA is involved in nicotine reinforcement. Further evidence of this has been shown by other disruptions to VTA dopaminergic output, such as lesioning the VTA projections to the nucleus accumbens, which also decreases nicotine self administration in rats (Corrigall et al., 1992).

Dopamine release in the nucleus accumbens is a characteristic of addictive drugs including nicotine: this has been demonstrated in the rat (Di Chiara and Imperato, 1988; Pontieri et al., 1996) and the rhesus monkey (Bradberry et al., 2000) using *in vivo* brain microdialysis, and in humans using PET neuro-imaging (Drevets et al., 1999; Drevets et al., 2001). Lesioning the nucleus accumbens can decrease nicotine self-administration, suggesting its involvement in nicotine reinforcement (Singer et al., 1982). The nucleus accumbens is comprised of two

anatomically distinct nuclei: the core and the shell (Heimer et al., 1991; Meredith et al., 1996). These nuclei have distinct limbic and cortical connections, suggesting that this region may be involved in integrating motivation and emotion (processes associated with the limbic system) with goal-directed behaviour (processes associated with the frontal cortex) (Zahm, 1999), which could contribute to addictive behaviours. Studies indicate that core and shell nuclei have different effects on dopamine release and nicotine reinforcement. Chronic non-contingent nicotine treatment increases dopamine concentrations in the core but not the shell (Cadoni and Di Chiara, 2000), while chronic self-administered nicotine increases dopamine concentrations in the shell but not the core (Lecca et al., 2006). Also, lesioning the core can increase impulsivity in rats (Cardinal et al., 2001), which is a contributing factor in addiction (de Wit, 2009), and increase nicotine conditioned place preference (Sellings et al., 2008), while lesioning the shell can decrease conditioned place preference (Sellings et al., 2008). Therefore, specific structures within the nucleus accumbens may be involved in different processes that contribute to nicotine reinforcement.

The prefrontal cortex is thought to modulate VTA activity through its glutamate projections to the VTA (Schilstrom et al., 1998). Antagonists that inhibit glutamatergic N-methyl-D-aspartate (NMDA) receptors in the VTA reduce dopamine release in the nucleus accumbens from nicotine treatment (Schilstrom et al., 1998). Thus, activation of VTA dopamine projections to the prefrontal cortex might be one component of nicotine's actions in the VTA-nucleus accumbens pathway (Murase et al., 1993; Gao et al., 2007). The prefrontal cortex is responsible for executive functioning processes such as working memory and attention; these processes are known to be modulated by dopamine (Granon et al., 2000) and nicotine has been shown to enhance these behaviours (Levin et al., 2006). Working memory and attention are important components of learning and thus may contribute to the development and persistence of addictive behaviour with and without the drug (Di Chiara, 1999). Since nicotine injected into the VTA (Nisell et al., 1994) as well as the prefrontal cortex (Drew et al., 2000) can elicit dopamine release, these two mechanisms could influence these cognitive processes. Thus, nicotine's actions in the VTA as well as the prefrontal cortex may be involved in nicotine reinforcement and withdrawal.

Other brain regions can contribute to nicotine dependence. These include the amygdala (Marcinkiewcz et al., 2009), hippocampus (Levin et al., 2006), pedunculopontine tegmental

nucleus (Lanca et al., 2000), insula (Naqvi et al., 2007), and habenula (Baldwin et al., 2011). Many of these structures have connections to the VTA, nucleus accumbens and/or prefrontal cortex (Mogenson et al., 1980), where they might modulate their activity or contribute independently to nicotine reinforcement. For example, the habenula is involved in controlling nicotine intake, elucidated with α5 knockout mice (Fowler et al., 2011), suggesting that it contributes to inhibitory behaviour related to intake rather than the primary rewarding effect of nicotine. The involvement of many brain regions in nicotine dependence suggests that complex mechanisms may underlie its development.

2.2.3 Neurobiological changes following continued nicotine exposure

Nicotine acts on multiple nAChRs in the brain reward system to elicit its rewarding effect. While its action on this system mediates the primary reinforcing effect of nicotine, chronic nicotine exposure leads to persistent neurobiological changes which are believed to reinforce drug seeking behaviour. Increased dopamine release in the nucleus accumbens has been reported after chronic nicotine treatment (Cadoni and Di Chiara, 2000; Lecca et al., 2006). Chronic nicotine exposure also leads to up-regulation of nAChRs in the brain, which has been reported in various species. Greater nAChR density was found in the post-mortem brains of human smokers compared to non-smokers (Benwell et al., 1988; Breese et al., 1997) and multiple studies found greater nAChR density in mouse (Marks et al., 1983) and rat brain (Morrow et al., 1985; Schwartz and Kellar, 1985; Lapchak et al., 1989) treated with nicotine compared to saline. Increased receptor binding is also found in rodents after chronic nicotine self-administration (Donny et al., 2000; Parker et al., 2004). This up-regulation is thought to be a homeostatic response to receptor desensitization by nicotine: chronic nicotine leads to longer desensitization of nAChRs (Grady et al., 1994; Eilers et al., 1997; Fenster et al., 1999) which reduces the availability of nAChRs. Therefore, more receptors are expressed to compensate for the loss of available receptors. Central nAChR up-regulation may contribute to nicotine tolerance, which has been observed after repeated exposure to nicotine (Stolerman et al., 1974; Rosecrans et al., 1989). Reduced rewarding effects of nicotine at the same level of nicotine intake could result in increases in nicotine-seeking behaviour, which may reinforce smoking behaviour in humans.

2.3 Nicotine self-administration

Nicotine dependence has been investigated using different models of smoking behaviour, such as the conditioned place preference (CPP) (Tzschentke, 1998) and nicotine self-administration (NSA) paradigms (Rose and Corrigall, 1997), where NSA is more commonly used. In NSA, the subject learns to make a response in order to receive one administration of drug at a given dose. Intravenous (IV) self-administration is a valid model used to predict abuse liability of drugs, where known drugs of abuse can establish this behaviour (Collins et al., 1983). The IV route in NSA is considered to model the rapid delivery of nicotine to the brain that occurs with smoking (Corrigall, 1999; Hukkanen et al., 2005). NSA also models the reinforced, self-administered behaviour of smoking puffs of the cigarette, as the subject has to make the learned response in order to receive a dose of the drug. These measurements can provide information about patterns of intake, motivation, drug-seeking behaviour and dose-response relationships.

In animal models of NSA (Thomsen and Caine, 2001), catheters are implanted into one jugular vein for nicotine delivery. They undergo self-administration sessions in operant chambers, which are equipped with the response operandum (for example, a lever to press) in order to access nicotine. In these chambers, drug lines are attached to the jugular catheters and responses for the drug are recorded. This model can be used to systematically investigate the neurobiological mechanisms and factors that can influence nicotine dependence.

NSA can be established in humans (Henningfield et al., 1983; Harvey et al., 2004; Sofuoglu et al., 2007), non-human primates (Deneau and Inoki, 1967; Spealman and Goldberg, 1982; Sannerud et al., 1994; Le Foll et al., 2007), rats (Corrigall and Coen, 1989; Donny et al., 1995; Glick et al., 1996), and mice (Martellotta et al., 1995; Yan et al., 2012), demonstrating that nicotine reinforcement can occur in multiple species. Moreover, evidence suggests that the same neurobiological substrates are involved in NSA and smoking: drugs that alter nAChR activity (Spealman and Goldberg, 1982; Corrigall and Coen, 1989; Corrigall et al., 1994; Watkins et al., 1999) and the dopamine system (Corrigall and Coen, 1991; Corrigall et al., 1992) can alter NSA behaviour, and the nAChR up-regulation found in the post-mortem brain of human smokers can also be seen in the brain of rats that self-administer nicotine (Donny et al., 2000).

2.3.1 Acquisition

Smoking behaviour can be divided into three stages: acquisition, maintenance, and cessation, which can be modeled by NSA using specific schedules of reinforcement. Acquisition is the stage where animals learn the response that elicits nicotine delivery. This stage is thought to reflect the initiation of smoking behaviour as animals begin to respond for nicotine injections. Acquisition in NSA is usually established using a fixed ratio (FR) schedule of reinforcement, where the same number of responses are required to elicit one nicotine injection (Thomsen and Caine, 2001). This typically begins with a small response demand such as one response (FR1), in order for animals to learn the association between responding and receiving nicotine. Once animals have learned to respond at this schedule the response demand increases to FR2 or FR3 in order to demonstrate that continued responding is due to its association with receiving nicotine. Thus the response, such as a lever press, which itself is not rewarding, becomes a conditioned reinforcer where receiving the drug reward is contingent upon this behaviour.

Early studies in rats demonstrated that nicotine on its own cannot elicit stable self-administration (Lang et al., 1977; Hanson et al., 1979; Latiff et al., 1980; Smith and Lang, 1980; Cox et al., 1984); however, Corrigall and Coen (1989) were able to establish robust and stable dose-dependent responding for nicotine with the addition of conditions such as limited access, food deprivation, food training and secondary cues (a light and a tone) coupled with nicotine delivery. This and subsequent studies (Donny et al., 1995; Shoaib et al., 1997; Donny et al., 1998) have shown that NSA can be acquired with nicotine doses ranging from 3-90 µg/kg/IV in limited access schedules. Acquisition was established most consistently with a dose of 30 µg/kg/IV nicotine, thus the majority of NSA literature after these initial studies trained animals with this 30 µg/kg nicotine dose to establish NSA and much of the focus in later NSA studies was on factors that influenced maintenance behaviour or cessation after acquisition.

In one study that collected blood after rats self-administered nicotine, nicotine doses from 15-60 µg/kg/IV resulted in nicotine plasma levels between 40-150 ng/ml (Shoaib and Stolerman, 1999), which is on the higher end of the range found in human smokers (10-50 ng/ml) (Benowitz, 1990a). This suggests that the doses rats self-administer can result in similar systemic levels, although plasma nicotine levels are not directly comparable because rats had limited access to nicotine (2 hours) in the NSA experiment (Shoaib and Stolerman, 1999) compared to

human smokers who can smoke any time of the day. Plasma nicotine levels from extended access paradigms, which would provide similar access time to that seen in humans, have not been reported in the literature; however, these studies have reported similar levels of nicotine intake (0.2–1.5 mg/kg/day) (Cohen and George, 2013) to that in smokers (0.14-1.14 mg/kg/day) (Benowitz and Jacob, 1984). Thus, the doses that animals acquire self-administration result in similar nicotine intake to humans, which suggests that nicotine reinforcement in this experimental model could be similar to that seen in smokers.

2.3.2 Maintenance

Once animals have acquired NSA they have reached the maintenance phase. This is thought to reflect smoking behaviour in already dependent smokers (Rose and Corrigall, 1997). In NSA this is measured under the FR schedule of reinforcement. Typically maintenance occurs when responding at one schedule reaches stable levels of responding and thus can provide information on patterns of intake. Nicotine's dose-response curve for NSA is generated using data from the maintenance phase (Corrigall and Coen, 1989). This dose-response curve displays the average level of stable responding for nicotine at multiple nicotine doses. Nicotine has an inverted U-shape dose-response curve similar to other drugs of abuse in rats and monkeys (Rose and Corrigall, 1997; Le Foll and Goldberg, 2009), where lower doses fall along the ascending limb of the dose-response curve until there is a dose where peak responding occurs. Doses higher than this elicit lower responding for the drug, suggesting that animals titrate their intake to achieve similar levels of the drug. The U-shape of nicotine's dose-response curve is shallower in rats than other drugs of abuse, such as cocaine (Rose and Corrigall, 1997), which suggests that nicotine is partially titrated at doses near its peak dose of responding. Humans also show dose-dependent responses in IV NSA (Harvey et al., 2004) where higher doses elicited lower responding consistent with titration.

While the FR schedule can provide information about nicotine intake, it cannot measure the reinforcing and motivational strength of nicotine. To test this, another schedule of reinforcement is used once animals have reached the maintenance stage. The progressive ratio (PR) schedule of reinforcement involves progressively increasing the response demand required for each successive drug delivery (Richardson and Roberts, 1996; Arnold and Roberts, 1997). The increasing response demand to elicit a single injection of drug is thought to reflect the

motivational strength of the drug because if the drug is highly reinforcing than animals are expected to respond more to obtain it. Motivational strength is measured by the breaking point, which is the last ratio of responses completed by the animal that elicited drug delivery (Richardson and Roberts, 1996). This schedule can be maintained in NSA in rats (Donny et al., 1999) and monkeys (Le Foll et al., 2007). The dose-response curve for PR in rats displays increases in breakpoint with increasing nicotine doses between 20 and 90 µg/kg/IV (Donny et al., 1999), indicating that higher doses of nicotine are more reinforcing. This pattern of responding is consistent with other drugs of abuse such as amphetamine or cocaine, although the PR dose-response curve in these drugs are more fully characterized than nicotine and display an inverted U-shape curve (Richardson and Roberts, 1996). Thus, there is a fairly linear dose-response relationship for nicotine under PR for doses within the range used in Donny et al. (1999).

2.3.3 Cessation

The last stage that can be modeled by NSA is cessation. For drugs of abuse, cessation precipitates various behaviours such as withdrawal, craving and relapse, which contribute to dependence. The NSA model has two different experimental paradigms that are thought to represent these behaviours: extinction and reinstatement. Withdrawal is defined as a set of signs or symptoms that manifest after the cessation of drug (Henningfield et al., 2009). It cannot be directly measured with NSA behaviour and is better assessed by a another paradigm where a set of somatic behaviours (withdrawal symptoms) are quantified (Bauzo and Bruijnzeel, 2012); however, the persistence of drug-seeking behaviour in the absence of drug delivery, called extinction, considered a behaviour related to withdrawal and craving, can also be measured in the NSA model (Markou et al., 1993). Extinction behaviour has been characterized in rats trained at the commonly used 30 µg/kg/IV nicotine dose (Donny et al., 1995; Shoaib et al., 1997; Shram et al., 2008a), where the substitution of nicotine for saline upon responding for nicotine leads to decreased responding over time within and across sessions.

Once NSA behaviour is extinguished, the ability of re-exposure to the self-administered drug, or to other stimuli, to reinstate drug-seeking behaviour can be investigated. Reinstatement is considered a measure of drug relapse and models the return to drug seeking after a period of abstinence from nicotine exposure. NSA behaviour in rats can be reinstated by re-administering

nicotine injections, after extinction produced by saline substitution (Donny et al., 1995) or after administering a single priming dose of nicotine following extinction (Chiamulera et al., 1996; Shaham et al., 1997; Shram et al., 2008a). Consistent reinstatement has been observed with 0.15 and 0.3 mg/kg/SC priming doses of nicotine, but were mixed for reinstatement with IV priming doses of nicotine: doses lower than that administered during NSA significantly increased responding for nicotine in one study (Chiamulera et al., 1996) while another study found significant reinstatement at the NSA dose (Shaham et al., 1997). Shaham et al. (1997) compared SC and IV priming doses of nicotine and found that SC nicotine elicited larger responding for nicotine than IV. The reasons behind the larger response to SC priming are not clear.

NSA behaviour in rats can be reinstated after re-presentation of the nicotine-paired stimuli, such as a light or tone cue (LeSage et al., 2004; Liu et al., 2007; Liu et al., 2008; Clemens et al., 2010), suggesting that cues may have reinforcing properties. In addition to nicotine's primary reinforcing effect, it acts as a secondary reinforcer by strengthening the association of stimuli (cues) paired with its intake (Palmatier et al., 2007b; Caggiula et al., 2009). The non-selective nAChR antagonist mecamylamine can block cue-induced reinstatement (Liu et al., 2007), suggesting that cue reinforcement in NSA is mediated by nicotine acting on nAChRs. Consistent with this, smoking-related cues have been associated with craving and relapse in humans (Niaura et al., 1989; Payne et al., 1991; Droungas et al., 1995). Therefore, nicotine's secondary reinforcement of nicotine-paired cues could be a contributing factor in the development of nicotine dependence.

2.3.4 Factors that influence NSA

NSA acquisition is sensitive to changes in the parameters of self-administration. These factors will be discussed as they are important in the design of NSA experiments. Corrigall and Coen (1989) first demonstrated that specific conditions such as food deprivation, prior food training, presence of secondary cues and training dose can influence acquisition of NSA. Other factors such as session length (limited vs. extended) (Harris et al., 2008) and access (intermittent vs. continuous sessions) (O'Dell and Koob, 2007) can also influence NSA behaviour. The effect of secondary cues on acquisition has been studied extensively (Caggiula et al., 2001; Caggiula et al., 2002; Palmatier et al., 2006; Palmatier et al., 2007a), establishing that the presence of cues can increase responding for nicotine and may be important in reinstatement. Food training is

commonly used to facilitate acquisition in limited access NSA (Corrigall and Coen, 1989; Donny et al., 1995; Donny et al., 1998). Animals are placed in the operant box and learn to respond for a food reward. Once the response has met a certain response criteria animals are switched from receiving a food reward to receiving an infusion of the drug contingent upon active responding. Nicotine training dose can also facilitate acquisition, with higher doses increasing the proportion of animals that acquire the behaviour (Corrigall and Coen, 1989; Shoaib et al., 1997; Donny et al., 1998; Shram et al., 2008b).

Facilitating acquisition is useful for studies designed to investigate the NSA behaviour once it has already been established; however, if NSA acquisition behaviour itself is the main goal of investigation than these factors may confound the findings. Limited access NSA behaviour can be acquired without prior food training, which is commonly referred to as spontaneous acquisition (Shoaib et al., 1997; Diergaarde et al., 2008a; Shram et al., 2008b; Fattore et al., 2009; Clemens et al., 2010; Peartree et al., 2012). However, the majority of these studies do not report detailed acquisition data and those that do use doses 15 µg/kg and higher to investigate acquisition, which is near the peak of nicotine's dose-response curve (Corrigall and Coen, 1989). Only one study has looked at the effect of food training on NSA acquisition head-to-head (Clemens et al., 2010) but only one training dose was used (30 µg/kg), which animals will readily acquire (Corrigall and Coen, 1989; Donny et al., 1995; Shram et al., 2008b). Furthermore, NSA acquisition can be rat strain dependent (Shoaib et al., 1997), therefore characterization of these factors are important in NSA study design. This demonstrates the utility of NSA in investigating the various aspects of nicotine dependence.

2.4 Nicotine metabolism

2.4.1 Human

Nicotine is metabolized primarily in the liver with a half-life of two hours (Hukkanen et al., 2005). A variety of metabolites are generated by nicotine metabolism in the liver (Figure 1). Cotinine is the main metabolite, where 70-80% of nicotine is converted into this product in humans. CYP2A6 is the primary enzyme responsible for metabolizing nicotine into cotinine (Nakajima et al., 1996b). It accounts for ~90% of cotinine formation through C-oxidation of nicotine's pyrrolidine ring (Al Koudsi et al., 2010). The enzyme metabolizes nicotine to a

nicotine-$\Delta^{1'(5')}$-iminium ion, which then gets converted to cotinine by aldehyde oxidase (Hukkanen et al., 2005). CYP2A6 can also metabolize nicotine through N-demethylation on the pyrrolidine ring to form nornicotine, which accounts for 1-3% of product formed from nicotine (Kyerematen et al., 1990; Yamanaka et al., 2004; Yamanaka et al., 2005). Cotinine can be metabolized further by CYP2A6 into trans-3'-hydroxycotinine (Nakajima et al., 1996a) which is a major metabolite found in urine, as well as norcotinine, and 5'-hydroxycotinine (Murphy et al., 1999).

CYP2B6 binds to nicotine with a lower affinity (K_m = 820 μM) than CYP2A6 (K_m = 144 μM) and has the second highest C-oxidation activity in the liver (Yamazaki, 1999; Dicke et al., 2005). CYP2B6 can also N-demethylate nicotine to form nornicotine (Yamanaka et al., 2005). Thus, CYP2B6 can contribute to nicotine metabolism but to a lesser extent than CYP2A6. It is possible that CYP2B6 could play a larger role in nicotine metabolism when CYP2A6 activity is absent or reduced

Other enzymes contribute to nicotine metabolism in the liver. Flavin-containing monooxygenase 3 (FMO3) produces nicotine-N-oxide from nicotine, which accounts for 4-7% of nicotine in urine (Benowitz et al., 1994). Uridine diphosphate-glucuronosyltransferase (UGT) enzymes glucuronidate nicotine into N-quarternary glucuronides, which accounts for 3-5% of nicotine in urine (Benowitz et al., 1994). The UGTs 2B10, 2B7, and 1A4 can glucuronidate nicotine, with 2B10 suggested to contribute the most to glururonidation (Nakajima et al., 2002; Kuehl and Murphy, 2003; Kaivosaari et al., 2007).

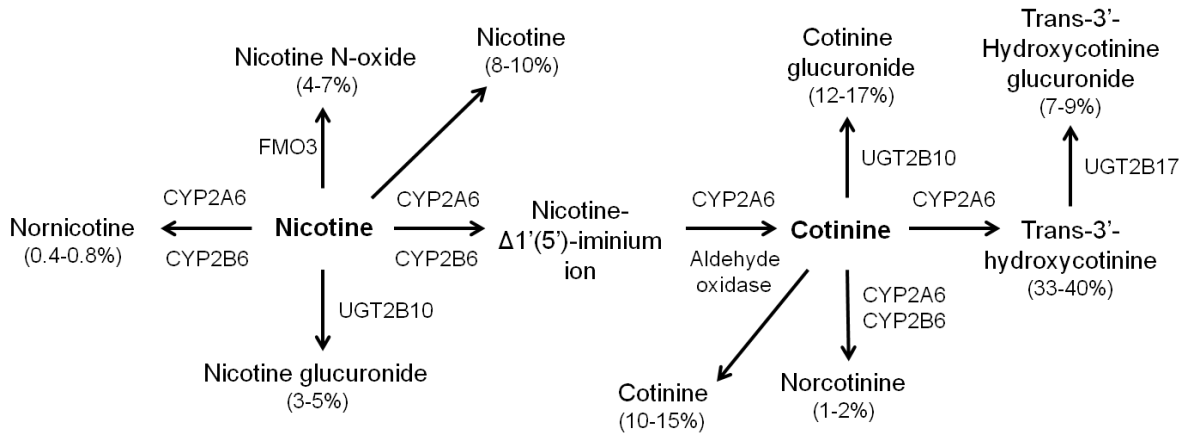

Figure 1. Nicotine metabolites found in human urine. Percentages are based on concentration of total urinary nicotine measured from smokers accompanied by the enzyme responsible for producing the metabolite. Figure is a modified version with the relevant CYP-produced metabolites from Hukkanen et al. (2005), which was compiled from multiple studies.

2.4.2 Rat

Nicotine is metabolized by the CYP2B6 homolog CYP2B1 in rat liver (Nakayama et al., 1993). It has a shorter half-life of one hour in rats compared to humans (Kyerematen et al., 1988a) and has greater affinity to CYP2B1 (K_m = 5-7 µM) (Hammond et al., 1991) compared to CYP2B6 (K_m = 820 µM) (Dicke et al., 2005). CYP2B1 is the primary enzyme that metabolizes nicotine in rat liver as CYP2A is not active towards nicotine in this species (Hammond et al., 1991). Although the main enzyme involved in nicotine inactivation is different in rats compared to humans, cotinine is still one of the major metabolites formed by CYP2B1 (Kyerematen et al., 1988b) and other nicotine metabolites found in humans can be quantified in rats (Table 2), such as nicotine-N-oxide, 3'-hydroxycotinine, nornicotine, and norcotinine (Kyerematen et al., 1988a; Kyerematen et al., 1988b; Craig et al., 2014), suggesting that nicotine can undergo similar metabolic reactions in both species. FMO1 in rat liver can N-oxidate nicotine (Katchamart et al., 2000); however, nicotine glucuronidation has not been reported in the rat.

Table 2. Nicotine metabolites found in rat urine

Metabolites recovered 120 hours following ^{14}C-Nicotine administration	
Metabolite	**Recovery (% of nicotine dose)**
Nicotine	11.3 ± 0.9
Cotinine	7.2 ± 0.9
Nicotine-1'-*N*-oxide	11.6 ± 0.9
Nornicotine	8.9 ± 0.9
3-hydroxycotinine	0.5 ± 0.6
3-hydroxycotinine glucuronide	N.D.

Table is a modified version taken from Kyerematen et al. (1988b).
Data was generated from one intra-arterial injection of 0.1 mg/kg of ^{14}C-Nicotine. Each metabolite was quantified as a percentage of the radioactivity recovered from urine.
N.D. = Not detected

2.4.3 Role of nicotine metabolism in smoking behaviour

2.4.3.1 Peripheral nicotine metabolism

CYP2A6 activity is considered an important contributor to smoking behaviour because it is responsible for metabolizing nicotine and thus regulating plasma nicotine levels. Genetic variation in the *CYP2A6* gene can alter plasma nicotine levels, where variants that reduce CYP2A6 activity result in higher plasma nicotine levels (Nakajima et al., 2000; Rao et al., 2000). Smokers with variant alleles that reduce CYP2A6 activity, identified as slow metabolizers, are less likely to be tobacco-dependent and smoke fewer cigarettes per day compared to normal metabolizers (Pianezza et al., 1998; Rao et al., 2000; Schoedel et al., 2004; Malaiyandi et al., 2006). While *CYP2A6* slow metabolizers are less likely to be dependent in adulthood, adolescent *CYP2A6* slow metabolizers have a greater risk of becoming dependent (O'Loughlin et al., 2004). This appears opposite of what would be expected based on what is known of adult slow metabolizers; however, adolescent slow metabolizers are more likely to quit (Chenoweth et al., 2013), suggesting that although they may find initial experiences with cigarettes rewarding, regular smoking behaviour might not be as reinforced compared to normal metabolizers. Also, although the risk of becoming dependent is greater in adolescent slow metabolizers, they convert to dependence at a slower rate compared to normal metabolizers (Audrain-McGovern et al., 2007) and are more likely to quit unaided (Patterson et al., 2008; Ho et al., 2009), which is consistent with smoking behaviour in adult *CYP2A6* slow metabolizers.

The nicotine metabolites cotinine and nornicotine can activate nAChRs and elicit dopamine release in rat striatal slices (Dwoskin et al., 1993; Teng et al., 1997; Dwoskin et al., 1999). Both metabolites can also alter behaviour: cotinine was recently shown to be capable of reducing depressive behaviours and working memory deficits in a chronic stress mouse model (Grizzell et al., 2014), while nornicotine can be self-administered by rats (Stairs et al., 2007) and its pre-treatment can reduce NSA (Green et al., 2000). Therefore, it is possible that these metabolites might be involved in smoking behaviour, which further supports the potential impact of altered nicotine metabolism in nicotine dependence.

2.4.3.2 Central nicotine metabolism

While the contribution of CYP2B6 to nicotine metabolism in the liver is small, < 10 % of cotinine formation (Al Koudsi and Tyndale, 2010), it is possible that it may play a greater role in the brain. CYP2B6 mRNA and protein are expressed throughout the brain while only mRNA from CYP2A6 has been detected in brain (Nishimura et al., 2003; Yamanaka et al., 2005). The presence of CYP2B6 and not CYP2A6 protein in the human brain, along with the evidence that *CYP2B6* genetic variation can influence smoking behaviour suggests that altered central nicotine metabolism could influence nicotine drug response. The rat model of CYP2B variation in the brain using MBIs can test whether brain CYP2B activity can influence NSA behaviour.

Many of nicotine's metabolites, including cotinine and nornicotine, are present in the rat brain. Brain nicotine, cotinine, nornicotine, and norcotinine concentrations were detectable following a single acute peripheral SC injection of radio-labeled nicotine (Crooks et al., 1995; Crooks et al., 1997; Ghosheh et al., 1999). Ghosheh et al. (1999) determined the half-lives of these compounds in the brain, with 52, 333, and 166 minutes reported for nicotine, cotinine, and nornicotine, respectively. Among all of the metabolites quantified cotinine had the highest concentration, with nornicotine the second highest, and norcotinine was detectable but lower than the other two metabolites. Subsequent work by this group examined nicotine accumulation in rat brain with repeated nicotine administration, either through intermittent injections or continuous infusion (Ghosheh et al., 2001). Both administration paradigms displayed greater concentrations in the brain compared to the blood for nicotine and nornicotine, suggesting partitioning of these compounds into the brain (Ghosheh et al., 2001). Brain accumulation of nicotine and nornicotine exposes the brain to higher amounts of these compounds for longer periods of time, which

increases their potential actions in this tissue. For nicotine, higher concentrations in the brain can increase dopamine release (Cadoni and Di Chiara, 2000; Lecca et al., 2006) and its subsequent rewarding effects. For nornicotine, higher concentrations in the brain can also increase dopamine release (Dwoskin et al., 1993; Teng et al., 1997), which might contribute to the rewarding effects of nicotine (Di Chiara, 1999). Nicotine and nornicotine can activate nAChR subtypes (Papke et al., 2007), and given that nornicotine has a longer retention time in the brain (Ghosheh et al., 1999), it is possible that that accumulation of these compounds could have different effects within the brain and that both may play a part in nicotine dependence.

Work from our group has also identified these metabolites in rat brain with the additional quantification of nicotine-N-oxide and *trans*-3'-hydroxycotinine (Craig et al., 2014). The results were consistent with the previous studies: after SC nicotine injection cotinine brain levels were highest followed by nornicotine. Nicotine-1'-*N*-oxide levels were lower than nornicotine but higher than norcotinine and *trans*-3'-hydroxycotinine levels were below the limit of quantification (Craig et al., 2014). Brain nicotine and metabolite levels after an IV injection were also measured. They displayed the same order of metabolite levels, where cotinine levels were the highest, followed by nicotine-1'-*N*-oxide and nornicotine, but overall levels of nicotine and the metabolites were lower compared to after SC injection, which was five times higher (1.0 mg/kg) than the IV nicotine dose (0.2 mg/kg) (Craig et al., 2014). Brain pharmacokinetic parameters from this study and the Ghosheh group are summarized in Table 3. These experiments demonstrate that nicotine metabolites are detectable in the brain and can accumulate over exposure, which could indicate local metabolism in this organ or the entry of metabolites produced from the periphery into the brain.

There is evidence that nicotine metabolism can occur in the brain: cotinine was detected from guinea pig and rat whole brain homogenates incubated with nicotine (Jacob et al., 1997) and human brain microsomes from striatum can N-demethylate nicotine (Yamanaka et al., 2005). Furthermore, when brain homogenates were pre-treated with the CYP2B inducer phenobarbital in the study by Jacob et al. (1997), cotinine formation was higher compared to homogenates not treated with the inducer, suggesting that CYP2B could be involved in cotinine formation. Rat brain microsomes can metabolize the CYP2B probe substrate pentoxyresorufin through 7-pentoxyresorufin-O-dealkylation (PROD) (Parmar et al., 1998; Dhawan et al., 1999), and PROD activity can also be increased with phenobarbital treatment, providing further evidence that the

increase in cotinine formation following phenobarbital treatment might be due to induced CYP2B activity.

Table 3. Pharmacokinetic parameters of nicotine and metabolites in the brain after a single injection of nicotine

SC nicotine administration			
Parameter	Nicotine	Cotinine	Nornicotine
$t_{1/2}$ (minutes)	52[a] 97[b]	333[a] 347[b]	166[a] 112[b]
Ratio (Brain:Blood)	4:1[c] 2:1[b]	1:1[c] 1:2[b]	2:1[c] 2:1[b]

a: Ghosheh et al. (1999); 0.8 mg/kg/SC nicotine
b: Craig et al. (2014); 1.0 mg/kg/SC nicotine
c: Ghosheh et al. (2001); 0.3 mg/kg/SC nicotine

2.4.3.3 *In vivo* nicotine microdialysis

Demonstrating that altered CYP2B activity in brain, but not liver, can change brain nicotine levels *in vivo* would provide evidence that variation in brain-specific drug metabolism has a functional impact. Nicotine is administered peripherally in both IV NSA in rats and in humans by smoking; therefore using *in vivo* techniques to measure brain levels after altering CYP2B activity would provide direct information on the central distribution of nicotine following peripheral administration of the drug. Also, the other processes involved in CYP2B-mediated metabolism are intact so metabolism would be expected to occur normally. Brain microdialysis is one technique by which the free levels of the compound can be measured (Ungerstedt, 1991). While the measurement of endogenous compounds like neurotransmitters has been well established with brain microdialysis (Kennedy, 2013), the measurement of drug levels with this technique is still fairly uncommon. To date, three brain microdialysis studies exist for nicotine. Chang et al. (2005) was the first study to report nicotine and cotinine levels in the brain via microdialysis. Rats were anaesthetized during microdialysis, where animals received one IV

injection of nicotine (2 mg/kg). While plasma levels of nicotine and cotinine were detected, only brain levels of nicotine were detected in the striatum and recovery of nicotine and cotinine was very low (3.2 and 3.4 %, respectively). Nicotine levels in the brain were highest at the first collection timepoint (10 minutes) and dropped rapidly. Given that metabolites can be detected in brain tissue (Crooks et al., 1995; Crooks et al., 1997; Ghosheh et al., 1999), it is possible that cotinine was below the limit of detection for the assay due to its low recovery by the probe or that cotinine does not circulate through the cerebrospinal fluid.

A second study by Woods et al. (2006) measured nicotine and cotinine levels in the nucleus accumbens and the lateral ventricle (ICV). Rats were awake and freely moving during microdialysis and received a single 0.5 mg/kg/IV dose of nicotine. This study did not report probe recovery of nicotine, however nicotine levels in both brain regions were detectable, showing a similar pattern over time to that in Chang et al. (2005), where nicotine levels were highest at the first collection timepoint (30 minutes). Finally, cotinine levels were detectable but low, which is consistent with that seen in Chang et al (2005).

The third study examined nicotine brain levels after a single SC and intragastric (IG) injection. Katner et al. (2014) were able to detect nicotine and cotinine levels in the nucleus accumbens following nicotine injection by either route of administration. Brain nicotine levels measured after three ascending doses of SC nicotine increased dose-dependently; however, there was no significant difference in nicotine levels between the two lower doses while the highest dose tested had significantly greater nicotine levels than these two doses. Peak brain nicotine levels were reported at 20 minutes post-injection, which appears later than that seen in the two IV studies (Chang et al., 2005; Woods et al., 2006); however, they all used different time frames for drug collection (10 or 30 minute collection periods for IV nicotine vs. 15 minutes for SC nicotine) so the results are difficult to compare. Brain cotinine levels were low, consistent with the two IV studies (Chang et al., 2005; Woods et al., 2006), but did show dose-dependent increases where the highest dose was significantly different from the two lower doses. Brain nicotine levels after IG administration were lower than SC at the same nicotine dose, possibly due to first-pass (ie. hepatic metabolism as nicotine enters the systemic system) metabolism of nicotine. Brain cotinine levels after IG administration were slightly higher than SC, which is in agreement with production of cotinine from first pass effects.

Together, these results demonstrate that brain nicotine levels can be measured by microdialysis and that brain nicotine levels can vary depending on the route of peripheral administration. The IV studies, however, only tested one nicotine dose each; therefore characterization of the dose-dependency in nicotine brain levels after IV injection needs to be determined. Therefore, the rat model of altered CYP2B activity in the brain, in addition to testing the effects of altered brain CYP2B activity on NSA, can also be used in microdialysis to determine whether it can influence the levels of nicotine in the brain.

Section 2: Materials and Methods

1 Effect of operant food training and training dose of nicotine on intravenous nicotine self-administration

1.1 Animals and housing conditions

Adult male Wistar rats (250-300 g) were obtained from Charles River Laboratories (Quebec, Canada) and individually housed in a temperature-controlled environment on a 12 hr reverse light/dark cycle (lights on at 1900 hr). Water was available ad libitum and 20-25 g of Purina rat chow was given daily after each operant session throughout nicotine self-administration.

1.2 IV catheter implantation surgery

Rats were anaesthetized with Isofluorane and the local anaesthetic Marcaine (Hospira Healthcare, Montreal, QC, Canada) was applied to incision sites (0.1 ml, 0.125%, SC). Derapen (Wyeth Animal Health, Guelph, ON, Canada; 0.1 ml, SC) was used as an antibiotic and Anafen (Merial Canada, Baie D'Urfé, QC, Canada; 5 mg/kg, SC) was used as an analgesic; both were given after rats were anaesthetized. Catheters were implanted into the right jugular vein as previously described (Shram et al., 2008b) and rats were given 7 days to recover before nicotine self-administration (NSA). To maintain catheter patency, IV catheters were flushed daily with Heparin in a sterile saline solution (0.1 ml, 50 U/ml) before and after each NSA session.

1.3 Self-administration apparatus

NSA was carried out in sixteen operant chambers that were operated by a computer-controlled interface system (Med Associates, St Albans, VT) under operational conditions previously described (Shram et al., 2008a; Shram et al., 2008b). Each operant chamber was set up with a grid floor and two levers on the same wall. Pressing on the active lever activated a high-speed microlitre syringe pump which delivered nicotine (0.1 ml/kg in approximately 1 sec). Pressing on the inactive lever was recorded but did not have any programmed consequences. A white cue light and a tone generator were located above the active lever. A white cue light was also located above the inactive lever at the same height as the light above the active lever; however pressing on the inactive lever did not turn this light on. Both visual (40 sec) and auditory (2800 Hz, 1 sec) stimuli above the active lever were turned on when nicotine was delivered. There was a timeout period (40 sec) following nicotine infusion, where lever presses were recorded but there were no consequences. Active lever presses reported in these experiments include all lever presses made during the session including those during the timeout period. On the top centre of the opposite wall of the chamber was a house light which signaled the start of the self-administration session. Nicotine was delivered via a modified 22-gauge cannula connected to a fluid swivel with Tygon tubing protected by a metal spring, which was attached to the intravenous catheter during each session. The swivel was connected to the microlitre syringe with Tygon tubing.

1.4 Drug concentration and preparation for NSA

Nicotine (Sigma-Aldrich, Oakville, ON, Canada) was prepared daily using sterile saline and pH was adjusted to 6.8–7.2. The unit doses for NSA were 3.75, 7.5, 15, and 30 µg/kg/infusion (nicotine expressed as base). For reinstatement tests, nicotine was administered subcutaneously in a volume of 1 ml/kg at 0.15 and 0.3 mg/kg. Thiopental (2-4 mg, IV, 20 mg/ml) was used to test catheter patency at the end of each experiment phase.

1.5 Self-administration procedures

1.5.1 Nicotine self-administration in multiple separate cohorts

1.5.1.1 Animal cohorts that underwent acquisition of NSA and PR with prior food training

Animals underwent NSA with prior food training in two separate cohorts with identical procedures. The first cohort self-administered 7.5, 15, and 30 µg/kg/infusion unit doses of nicotine and the second cohort self-administered 3.75 µg/kg/infusion unit dose of nicotine (Figure 2a) (Garcia et al., 2014). Before surgery, rats (n = 12/nicotine infusion dose) underwent operant training for 45 mg sucrose pellets (Bioserv, Frenchtown, NJ) in operant chambers equipped with pellet magazines. All components of the operant chambers (active and inactive levers, house and cue lights) were set up in the same locations as they are for NSA, with the exception of the pellet magazines which were on the same wall between active and inactive levers. Rats were food deprived for 24 hrs prior to the first food training session. Food training was conducted under a fixed ratio 1 (FR1) schedule of reinforcement in two 8 hr sessions with no audio or visual cues presented. Subsequently, responding for food was assessed in a 1 hr session in which rats were considered successfully trained once they received 100 pellets within the session.

Rats were assigned to one nicotine infusion dose throughout self-administration. Rats initiated 1 hr NSA sessions under an FR1 schedule for five days and then under an FR2 schedule for five days. Rats underwent each schedule Monday to Friday and were in their home cages Saturday and Sunday. Following FR training, rats then underwent a progressive ratio (PR) schedule of reinforcement. The sequence was determined using the exponential formula ($5^{((0.2 \times \text{infusion number}) - 5)}$), such that the required responses per infusion are as follows: 3, 6, 10, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 179, 219, 268, 328, 402, 492, 603 (Depoortere et al., 1993; Donny et al., 1999; Shram et al., 2008b). Self-administration parameters during the PR sessions were identical to FR training with the exception of session duration (2 hr) and continuous daily self-administration sessions (no days off). Animals achieved breakpoint (final ratio completed of active lever presses) when there was >20 minutes of inactivity on the active lever or until the session ended. Animals from these experiments were labeled food trained animals (FT).

1.5.1.2 Animal cohorts that underwent spontaneous acquisition of NSA and PR

Animals underwent NSA without food training in three separate cohorts with identical procedures. The first cohort self-administered 3.75 µg/kg/infusion of nicotine, the second cohort self-administered 7.5 and 15 µg/kg/infusion, and the third cohort self-administered 30µg/kg/infusion (Figure 2b) (Garcia et al., 2014). Rats initiated spontaneous NSA under the same conditions as rats that were food trained (five days under FR1 and FR2 and then PR) with the following exceptions: (1) animals did not receive food training and self-administration sessions commenced one week following recovery from surgery, (2) session duration was 2 hr for FR, and (3) sessions were conducted on a daily basis. Animals that underwent these experiments were labeled spontaneous acquisition rats (SP) because they did not receive training in the operant boxes prior to the beginning of NSA.

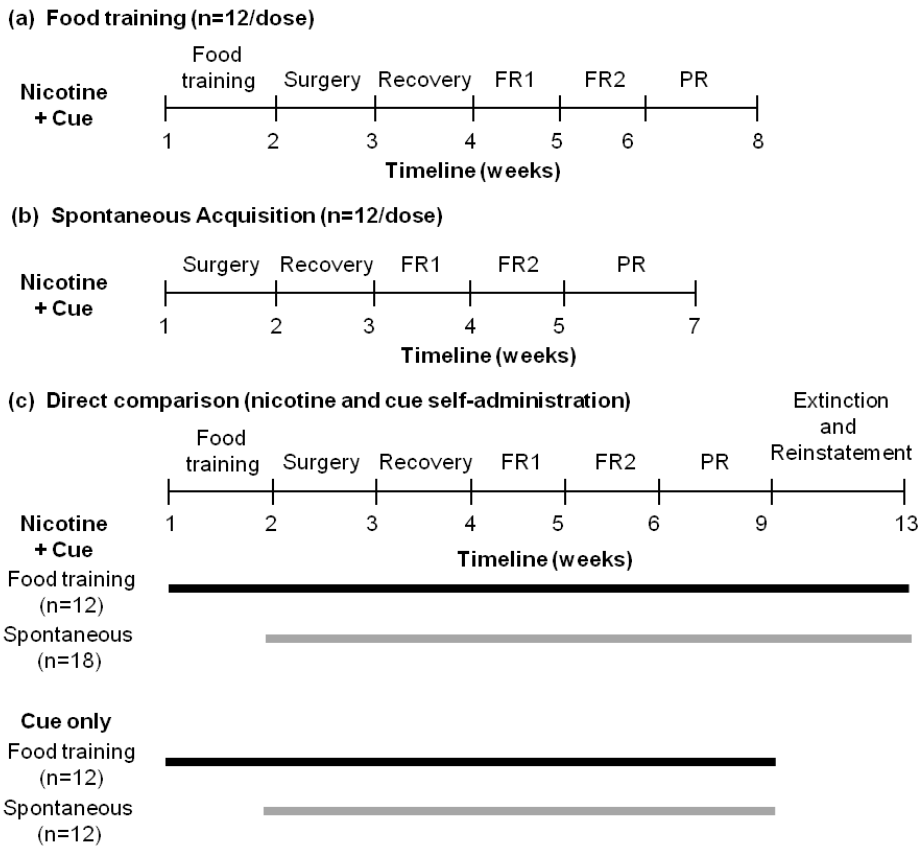

Figure 2. Study design for the food training and spontaneous acquisition self-administration experiments. There were two separate training approaches and a direct comparison of the training approaches as follows: **(a)** Animals were food trained and had 1 hr sessions that were carried out Monday to Friday; **(b)** Animals underwent spontaneous acquisition and had 2 hr sessions that were carried out continuously; **(c)** Animals were either food trained or spontaneously acquired nicotine self-administration or underwent cue self-administration in a direct head-to-head comparison and had 2 hr sessions that were carried out continuously. All other self-administration parameters were identical in all three experiments.

1.5.2 Nicotine self-administration in a head to head comparison of food training and spontaneous acquisition

1.5.2.1 Acquisition, PR and extinction and reinstatement of NSA in animals with and without food training

Animals were assigned to two groups: food trained (FT) and spontaneous acquisition (SP) (Figure 2c) (Garcia et al., 2014). Animals in the FT group underwent operant training for sucrose pellets before surgery as described in section 1.5.1.1. SP animals were not exposed to the operant boxes until after surgery. After surgery and recovery, both FT and SP initiated NSA under the same conditions described in section 1.5.1.2 for FR1 and FR2, where all animals were trained on 7.5 µg/kg/infusion nicotine. Following the last session of FR2, animals were switched to a PR schedule with the same infusion dose for five daily sessions. To determine whether each group responded differently for nicotine at higher nicotine infusion doses, the infusion dose was increased to 15 µg/kg for five PR sessions and then 30 µg/kg for another five PR sessions.

After PR the animals were returned to an FR2 schedule for 3 days and were then tested for extinction and reinstatement. During the extinction sessions, responding on the active lever did not elicit nicotine infusions or the presentation of auditory and visual cues that were previously associated with nicotine delivery. Animals underwent daily extinction sessions until they reached extinction criteria, which consisted of <20 active lever presses in two consecutive sessions. Once criteria were met a cue-induced reinstatement within a single self-administration session was conducted, where responding on the active lever resulted in the delivery of the compound cues (visual and auditory) that were previously associated with nicotine delivery. Additional extinction sessions were then carried out until they met extinction criteria again and responding on the active lever (nicotine reinstatement) after priming injections of saline, 0.15 and 0.3 mg/kg of nicotine (given 10 minutes prior to the start of the session) were then evaluated in three subsequent self-administration sessions using a repeated measure design.

1.5.2.2 Acquisition of self-administration behavior in response to the compound cue

Acquisition of operant responding for the compound cue without nicotine delivery in FT or SP rats was examined in two separate groups (n=12 each) (Figure 2c) (Garcia et al., 2014). Both FT and SP animals underwent operant self-administration under the identical conditions used for NSA (as described above in the FT and SP head-to-head experiment in Section 1.5.2.1) with the

exception that animals did not receive nicotine infusions. Animals underwent FR1, FR2, and PR for 5 days under each reinforcement schedule.

1.6 Data and statistical analysis

Criteria for NSA acquisition consisted of: (1) A ratio of at least 2:1 active compared to inactive lever presses; (2) at least 10 lever presses in the majority of sessions (at least 3 out of 5); and (3) at least 10 reinforcements in the majority of sessions (at least 3 out of 5). These criteria were assessed based on data under the FR2 schedule and are adapted from Shram et al. (2008b).

Acquisition data were analyzed using Fisher's Exact tests (2-sided). Self-administration data from the animals that acquired were analyzed using repeated measures analysis of variance (ANOVA) tests to compare between doses and training paradigms. Post-hoc analysis was conducted using t-tests with the Bonferroni correction. Analysis of data from the PR schedule was conducted using the log transformation of final ratio completed. Significance level for all comparisons was $p = 0.05$.

2 Effect of brain CYP2B inhibition on intravenous nicotine self-administration behaviour

2.1 Animals and housing conditions

Adult male Wistar rats (250-300 g, Charles River Laboratories, Quebec, Canada) were housed individually on a reverse 12 hr light/dark cycle with food restrictions identical to Section 1.1.

2.2 ICV cannulation surgery

Animals were given anaesthesia and analgesics in procedures identical to IV catheterization in Section 1.2. Stereotaxic surgery was conducted where stainless steel guide cannula were implanted into the right lateral ventricle for ICV injection [anterior-posterior 0.9 mm, lateral 1.4 mm, and dorsoventral 3.6 mm from Bregma (Paxinos, 1986)]. Briefly, a small hole was drilled into the skull and the cannula was fixed in place with dental cement anchored with jewelers' screws. Dummy cannulas were inserted into the guide cannula after surgery.

2.3 IV catheter implantation surgery

Seven days after ICV cannula implantation, catheters were implanted into the right jugular vein in animals that underwent NSA, as previously outlined in Section 1.2. Catheter patency was tested at the end of each phase (FR, PR or reinstatement) using Thiopental (previously described in Section 1.4) and non-patent animal data was removed from analysis.

2.4 Drug concentrations and preparation for NSA

For brain CYP2B inhibition, C8-xanthate, (C8X, Toronto Research Chemicals, Toronto, ON, Canada) the CYP2B-specific inhibitor (Yanev et al., 2000), was injected ICV, 20 µg in 4 µl of artificial cerebrospinal fluid (ACSF) over 1 minute; the injector was removed after 1 minute. Injections of sterile ACSF or C8X were made with a 30-gauge injector connected to a 10-µL Hamilton syringe. This C8X dose, given ICV, was previously optimized to inhibit CYP2B in the brain but not the liver (Khokhar, 2011; Khokhar, 2012; Khokhar, 2014) and was retested here with nicotine as a substrate. Sterile nicotine solution (Sigma-Aldrich, Oakville, ON, Canada) was prepared on each experiment day as a concentration of nicotine base in saline, pH 6.8-7.2, identical to the preparation in Section 1.4. Thiopental and Heparin were administered under identical procedures described in Section 1.4.

2.5 Nicotine self-administration procedures

2.5.1 Acquisition of NSA

NSA was carried out in sixteen operant chambers operated by a computer-controlled interface system (Med Associates, St Albans, VT) under same conditions described in Section 1.3. Rats were assigned to receive either C8X or ACSF (n=12/dose) ICV injections daily 22 hours before each self-administration session. Seven days after IV surgery, animals underwent NSA without food training (Figure 3a) at three infusion doses (3.75, 7.5, or 15 µg/kg) that were found to fall along the ascending limb of nicotine's dose-response curve for spontaneous acquisition (experiment described in Section 1.5.1.1 and results reported in Section 3.1.1) (Garcia et al., 2015). Acquisition was determined from the FR schedule, where rats underwent 2 hr daily NSA sessions under a FR1 schedule for 5 days and then a FR2 schedule for another 5 days. Acquisition criteria were identical to those described in Section 1.5.3.

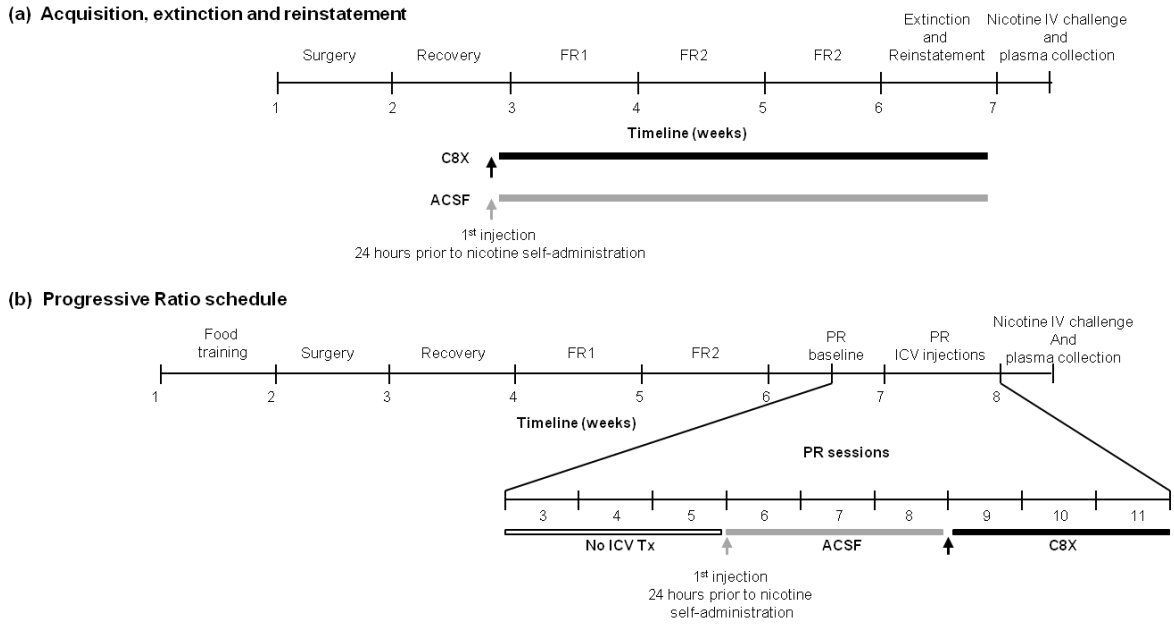

Figure 3. Study design for the brain CYP2B inhibition self-administration experiments. **(a)** Rats that underwent acquisition, maintenance, extinction, and nicotine reinstatement experiments under a fixed ratio (FR) schedule of reinforcement received daily ICV injections of ACSF or C8X. **(b)** Rats that underwent the progressive ratio (PR) schedule of reinforcement received a single ICV injection of ACSF and then C8X in a within-animal design. The No ICV Tx condition consisted of NSA sessions where there was no ICV pre-treatment.

2.5.2 Progressive ratio schedule

Prior to both ICV and IV surgery, rats were food trained to learn the self-administration operant behavior as previously described in section 1.5.1.1. Following recovery from IV surgery, rats (n=12/dose) underwent daily 1 hr NSA sessions under FR1 and FR2 for five days at a single nicotine dose of 7.5, 15, or 30 µg/kg (Figure 3b) (Garcia et al., 2015). Rats then underwent a PR schedule of reinforcement at their same training dose, in 2 hr daily sessions for 5 sessions to create a baseline of responding, as previously described in section 1.5.1.1. At 22 hours prior to the 6th PR session all animals received one ICV injection of ACSF then underwent three PR sessions (sessions 6, 7, and 8). At 22 hours prior to the 9th PR session all animals received one ICV injection of C8X then underwent three PR sessions (9, 10, and 11).

2.5.3 Extinction and reinstatement

Another cohort of rats (n=24 each for the C8X and ACSF groups) spontaneously acquired NSA with 7.5 µg/kg nicotine and underwent extinction and reinstatement. Following acquisition where rats were trained to self-administer nicotine and given daily ICV injections as described in Section 2.5.1 (Figure 3a) (Garcia et al., 2015), rats continued NSA under the FR2 schedule for 3 extra days and then underwent four 2 hr extinction sessions (one per day) during which responding on the active lever had no consequences. The effects of CYP2B inhibition on nicotine-induced reinstatement were then evaluated. On the reinstatement test day, rats were subjected to multiple 1 hour extinction self-administration sessions until they met extinction criteria (<10 active lever presses in two consecutive sessions). They were then given saline IV (0.1 ml/kg) 5 minutes before one 1 hour self-administration session. Following the saline self-administration session, reinstatement of nicotine seeking induced by different priming dose of nicotine (15, 30, and 60 µg/kg, 0.1 ml/kg IV) were evaluated in ascending order. Reinstatement tests were conducted over 3 days because of limited availability of operant chambers to run multiple sessions in one day (16 operant chambers). After one week, this extinction and reinstatement cycle was repeated with saline and nicotine (15, 30, 60 and 150 µg/kg SC).

2.5.4 Peripheral levels of nicotine and cotinine after NSA

Two different pharmacokinetic experiments were conducted at the end of the NSA behaviour. In the first experiment, after PR NSA rats from Section 2.5.2 received one ICV injection of ACSF or C8X 22 hrs before a bolus injection of nicotine (0.2 mg/kg/IV). Blood was collected from the saphenous vein at 15 and 60 minutes post-nicotine injection, and trunk blood was collected at 120 minutes. Plasma was analyzed for nicotine and cotinine by high performance liquid chromatography (Siu et al., 2006). In the second experiment, after nicotine reinstatement rats from Section 2.5.3 received four nicotine infusions over 4 minutes (7.5 µg/kg, one infusion per minute) in the operant chambers 22 hrs after the last ICV injection of C8X or ACSF. Trunk blood was collected at either 1 or 5 minutes after the last (fourth) infusion and resulting plasma was analyzed by liquid chromatography mass spectrometry (LC-MS) for nicotine and cotinine (Vieira-Brock et al., 2013; Craig et al., 2014). LC-MS was used to analyze plasma collected after the four infusions of nicotine because the total dose of nicotine was low (30 µg/kg); therefore, this was a more sensitive method to use to detect nicotine levels.

2.6 Data and statistical analysis

Acquisition data between C8X and ACSF ICV treatments were analyzed using a Fisher's Exact test (2-sided). Active and inactive lever presses and reinforcements in FR as well as the log-transformation of final completed ratios in PR within and between ICV treatments were analyzed with repeated measures ANOVA. Post-hoc analyses for all ANOVAs were conducted using t tests with Bonferroni correction. Extinction sessions (multiple sessions on the reinstatement test day) within and between C8X and ACSF ICV treatments were analyzed using Kaplan-Meier survival analysis followed by a log-rank test. Significance level for all comparisons was $p = 0.05$.

3 Effect of brain CYP2B inhibition and induction on nicotine brain levels measured by *in vivo* microdialysis

3.1 Animals and housing conditions

Animals that underwent *in vivo* microdialysis were individually housed after surgery on a 12 hr light/dark cycle. Water and food was provided *ad libitum*.

3.2 ICV cannulation surgery

Animals underwent surgery to implant guide cannula into the right lateral ventricle using identical procedures described in Section 2.2, with a few exceptions. The cannulas used (MD-2250; Bioanalytical Systems, Inc., West Lafayette, IN) were specific for the microdialysis probes (MD-2200; Bioanalytical Systems, Inc., West Lafayette, IN). Also, magnets were implanted in the dental cement cap to attach a metal spring for microdialysis tubing, and the Bregma coordinates for dorsoventral placement were different to allow for the 2 mm probe membrane to protrude from the cannula into the ventricle [anterior-posterior 0.9 mm, lateral 1.4 mm, and dorsoventral 2.6 mm from Bregma (Paxinos, 1986)]. Animals were given 7 days to recover before experiments commenced.

3.3 Drug concentrations and preparation for microdialysis

Nicotine was prepared on the same day as microdialysis under identical procedures as in Sections 1 and 2. The CYP2B inhibitor C8X and ACSF were also prepared and delivered ICV under identical procedures as Section 2 (ie. 22 hrs before nicotine administration).

3.4 *In vivo* nicotine microdialysis for brain CYP2B inhibition

For microdialysis (Figure 4a) (Garcia et al., 2015), Ringer's solution (147 mM Na^+, 2 mM Ca^{2+}, 4mM K^+, 155 mM Cl^-, pH 6.0) was perfused through concentric silica coated probes with 2 mm membranes (MD-2200; Bioanalytical Systems, Inc., West Lafayette, IN) by an infusion pump at 2 µl/minute. Baseline dialysate was collected for 15 minutes prior to IV nicotine injection into the tail vein, after which dialysate was collected on ice in 15 minute bins for 2 hours and 15 minutes. Blood (200-300 µl) was also collected from the saphenous vein at 15 and 75 minutes post-injection. For nicotine and cotinine quantification, 30 µl of dialysate were diluted to 100 µl with deuterium-labeled internal nicotine and cotinine standards (20 ng/ml in 0.01M HCl), while 100 µl plasma samples were prepared as previously described (Vieira-Brock et al., 2013; Craig et al., 2014); dialysate and plasma samples were then analyzed by LC-MS (Craig et al., 2014).

One cohort of animals (Figure 4b) (Garcia et al., 2015) was used to generate a dose-response curve for nicotine dialysate levels, where animals were given three different nicotine doses (0.15, 0.225 and 0.30 mg/kg, 1 ml/kg IV) and underwent microdialysis (No ICV Tx, n=5/nicotine dose). In another cohort of animals (Figure 4c) (Garcia et al., 2015) the effect of C8X was tested, where rats were given a single ICV injection of ACSF vehicle (n=10) or C8X (n=9) approximately 22 hrs prior to receiving one nicotine dose (0.15 mg/kg IV) and undergoing nicotine microdialysis.

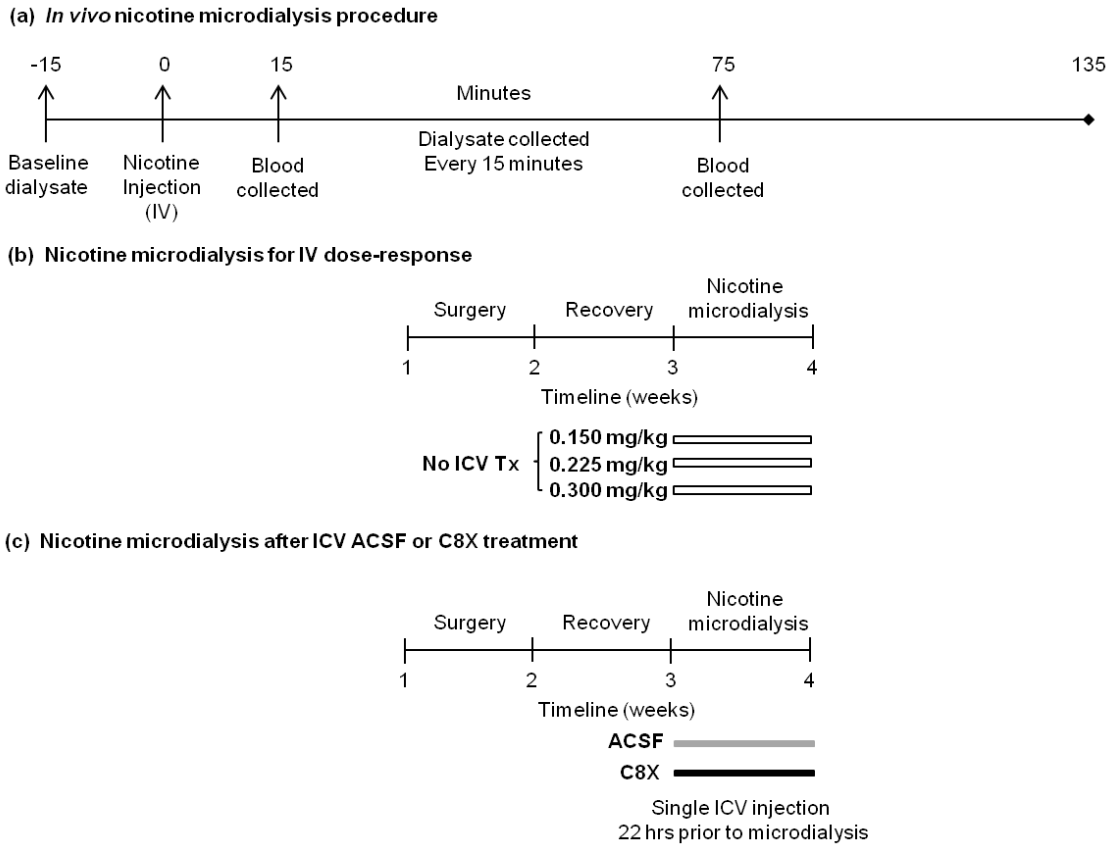

Figure 4. Study design for *in vivo* nicotine microdialysis following IV nicotine administration. **(a)** Microdialysis procedures for a single session. **(b)** Rats underwent a single nicotine microdialysis session at three different nicotine doses to generate a dose-response curve for brain nicotine levels. These animals did not receive ICV injections and are labeled "No ICV Tx." **(c)** Rats underwent a single nicotine microdialysis session at one nicotine dose (0.15 mg/kg) following pre-treatment ICV injection with ACSF or C8X.

3.5 *In vivo* microdialysis for brain CYP2B induction

Nicotine microdialysis procedures are identical to microdialysis in section 3.4 with the exception of SC nicotine delivery instead of IV (Figure 5a). A nicotine challenge injection for each microdialysis session was given SC at a dose of 1 mg/kg in the same volume (1 ml/kg). For brain CYP2B induction, nicotine (1 mg/kg/SC) was given once daily for seven days (Figure 5b). Nicotine microdialysis was conducted before and after this 7-day treatment, using the nicotine

challenge dose, which have been labeled acute and chronic conditions (Figure 5b). To determine whether the effect of CYP2B induction was maintained following cessation of nicotine treatment, animals were left in their home cages for 2 weeks, labeled as a washout, and then underwent a single nicotine microdialysis session with the same nicotine challenge dose for acute and chronic conditions (Figure 5c).

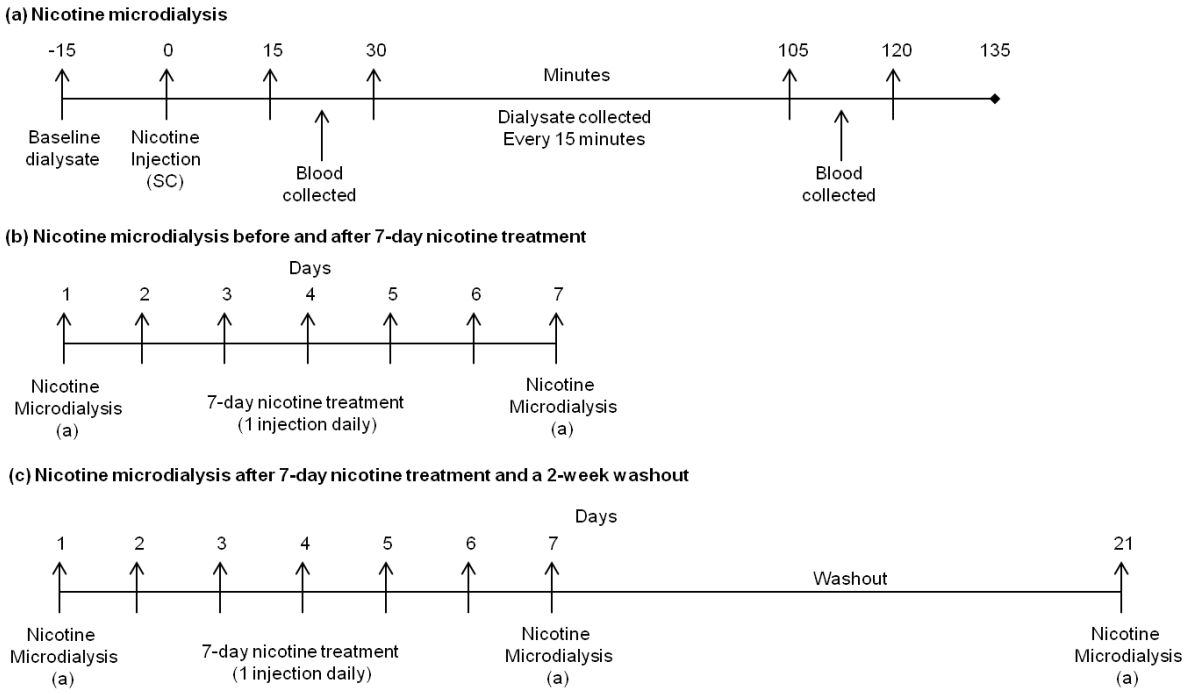

Figure 5. Study design for *in vivo* nicotine microdialysis following SC nicotine administration. **(a)** Microdialysis procedure for a single session. **(b)** Rats (n=20) underwent nicotine microdialysis following the first nicotine injection of the 7-day nicotine treatment (acute condition) and underwent nicotine microdialysis after the seventh nicotine injection (chronic condition). **(c)** After chronic nicotine microdialysis, rats had a 2-week washout period where they did not receive nicotine and underwent one last nicotine microdialysis session. Only a subset of rats (n=8) were able to complete the washout nicotine microdialysis session.

To establish that changes in brain nicotine levels were due to altering CYP2B activity, C8X was used. The effect of C8X ICV injection on brain nicotine levels following SC nicotine

administration was tested in a within-animal design where animals were given one ICV injection of ACSF 22 hrs before one acute nicotine microdialysis session and then given one ICV injection of C8X 22 hrs before a subsequent acute nicotine microdialysis session (Figure 6a). Another group of animals were used to examine the effect of inhibition on brain nicotine levels during induction. Animals underwent one nicotine microdialysis session on day 1 of the 7-day nicotine treatment (acute) and then one nicotine microdialysis session on day 7 of the 7-day nicotine treatment (chronic); however, these animals were split by ICV treatment where half of the rats were given daily ACSF ICV injections and the other half were given daily C8X ICV injections starting 22 hrs before the acute microdialysis session (Figure 6b). Finally, another group of animals underwent the same study design as animals in Figure 6a; however, after the chronic nicotine microdialysis session all animals were given one ICV injection of C8X the next day and then nicotine microdialysis was conducted the following day to examine whether inhibition would reverse the effect of CYP2B induction on nicotine levels in the brain (Figure 6c).

In all nicotine microdialysis sessions, because brain dialysate levels are a total of dialysate collected over a period of time (15 minutes), blood was collected in the middle of the 15 minute dialysate collection bins at minute 22.5 (mid-point for the 15-30 minute dialysate collection) and at minute 112.5 (mid-point for the105-120 minute dialysate collection) (Figure 5a). Plasma nicotine and cotinine levels were measured by LC-MS under the same conditions described in Section 3.4.

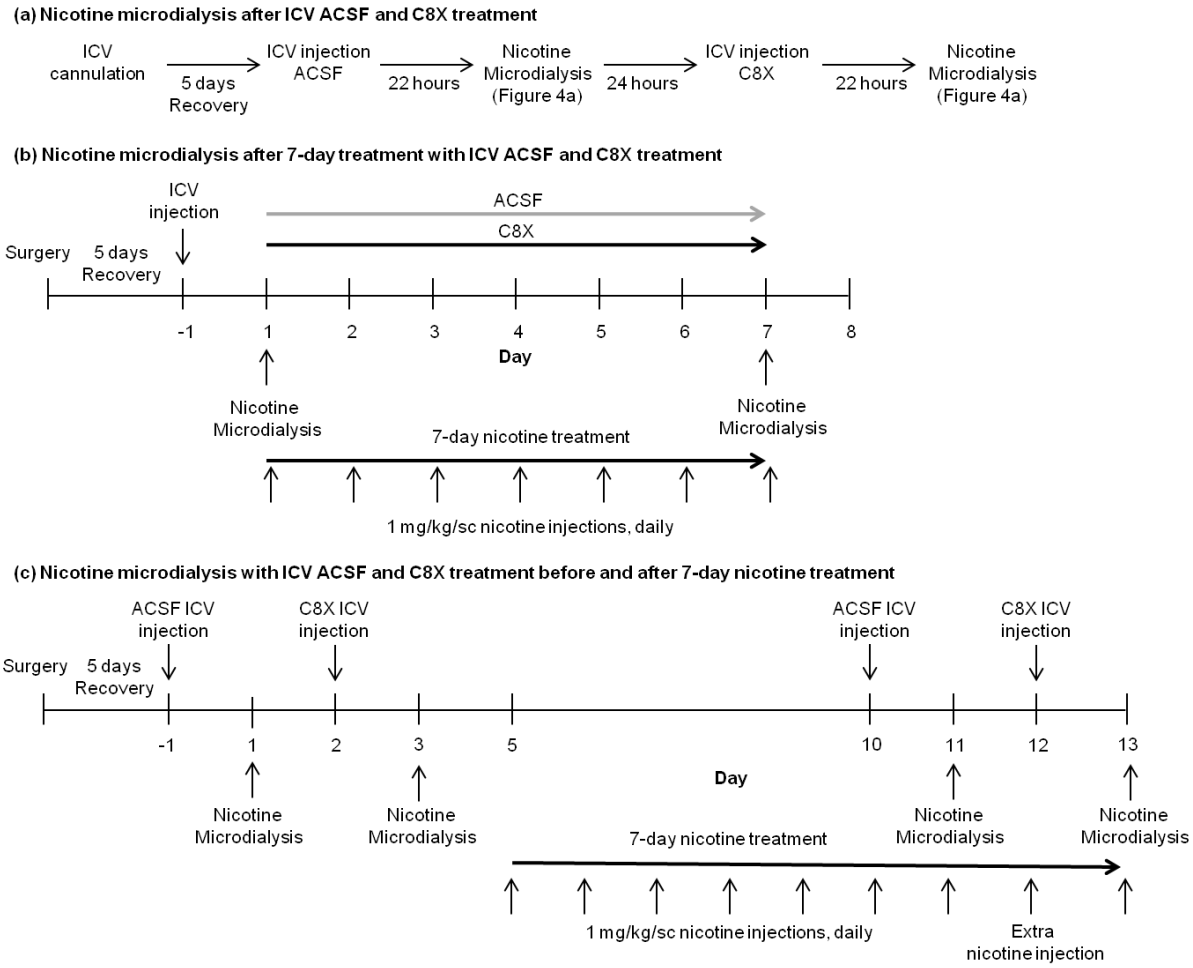

Figure 6. Study design for ICV pre-treatment of C8X in nicotine microdialysis following SC nicotine administration. **(a)** Nicotine naïve rats (n=16) were given an ACSF ICV injections 22 hours prior to their first microdialysis session and then given a C8X ICV injection 22 hours prior to another microdialysis session. **(b)** Nicotine naïve rats started ACSF (n=6) or C8X (n=8) daily ICV injections the day before their first nicotine microdialysis session (acute condition) and then underwent 7-day nicotine treatment. Following the 7th injection a second nicotine microdialysis (chronic condition) was carried out. **(c)** Rats (n=3) underwent 7-day nicotine treatment with the addition of within-animal ICV injections of ACSF and C8X after 7-day nicotine treatment. In order to maintain induction throughout microdialysis in the chronic condition, an 8th injection of nicotine at the same dose for 7-day treatment was given.

3.6 Data and statistical analysis

For brain CYP2B inhibition experiments, brain nicotine levels measured in the dialysate at 15-30 minutes post-injection, within 0-45 minutes and within 45-135 minutes after C8X and ACSF ICV treatment were analyzed by Student's t test. Plasma nicotine dose-response data was analyzed using a one-way ANOVA. For brain CYP2B induction experiments, brain nicotine levels at 15-30 and 15-45 minutes post-injection between acute and chronic conditions were analyzed by paired Student t tests. Nicotine and cotinine dialysate levels over time between acute and chronic conditions and between C8X and ACSF ICV treatments were analyzed using repeated measures ANOVA. Post-hoc comparisons were conducted using t tests with Bonferroni correction. Significance level for all comparisons was $p = 0.05$.

Section 3: RESULTS

1 Effect of operant food training and training dose of nicotine on intravenous nicotine self-administration

Nicotine self-administration behaviour is dependent on operant procedure parameters which include prior operant training, training dose, response operandum, and use of secondary reinforcers (Corrigall and Coen, 1989; Donny et al., 1998; Caggiula et al., 2001, 2002; Chaudhri et al., 2006). In order to investigate the role of altering brain CYP2B activity on nicotine self-administration, it is important to determine which parameters can influence the behavioral outcomes measured and optimize the procedure to ensure that any changes to these outcomes are due to our specific manipulation of brain CYP2B. Therefore, the effect of prior food training and training dose of nicotine was examined on acquisition, responding under a fixed-ratio schedule, and extinction and reinstatement of nicotine self-administration. The effect of food training and training dose was examined in four separate experiments where animals were either trained with food prior to nicotine self-administration or had no food training (spontaneous acquisition) at four nicotine infusion doses (3.75, 7.5, 15, and 30 µg/kg). Results modified version of Garcia et al. (2014) (doi: 10.1016/j.bbr.2014.07.043).

1.1 Dose-dependent acquisition of nicotine self-administration observed without prior food training compared to acquisition with prior food training

Acquisition was examined in rats that did not have prior food training (spontaneous, SP) and in rats that were food trained (FT) prior to nicotine self-administration at the four nicotine infusion doses. The percentage of rats that met acquisition criteria increased dose-dependently under SP conditions (Fisher's Exact, $p = 0.006$, Figure 7a). In contrast, approximately half of rats with prior FT (55%) acquired nicotine self-administration when trained at 3.75 µg/kg, while the majority of rats with prior FT (88 %) met acquisition criteria when trained at 7.5, 15, and 30 µg/kg doses of nicotine (Figure 7a), showing that FT enhanced acquisition at lower nicotine infusion doses.

In rats that met acquisition criteria, mean nicotine reinforcements for FT (1 hr sessions) animals showed an inverted U-shape dose-response curve with the highest mean reinforcements under the 15 µg/kg training dose ($F[3, 24] = 8.797$, $p < 0.001$) (Figure 7b). Mean reinforcements in SP animals (2 hr sessions) decreased with increasing infusion dose of nicotine both at 1 hr and at the end of session (ie. 2 hr, Figure 7b), but there was no significant difference between doses under this paradigm. These results show that both FT and SP acquired animals responded to receive similar amounts of nicotine at each infusion dose regardless of access time except for the lowest infusion dose (3.75 µg/kg), where SP rats earned half of their total infusions halfway through their NSA session. This suggests that once NSA was acquired responding was driven by the infusion dose, where SP rats had double the time to earn the same amount of reinforcements as FT rats.

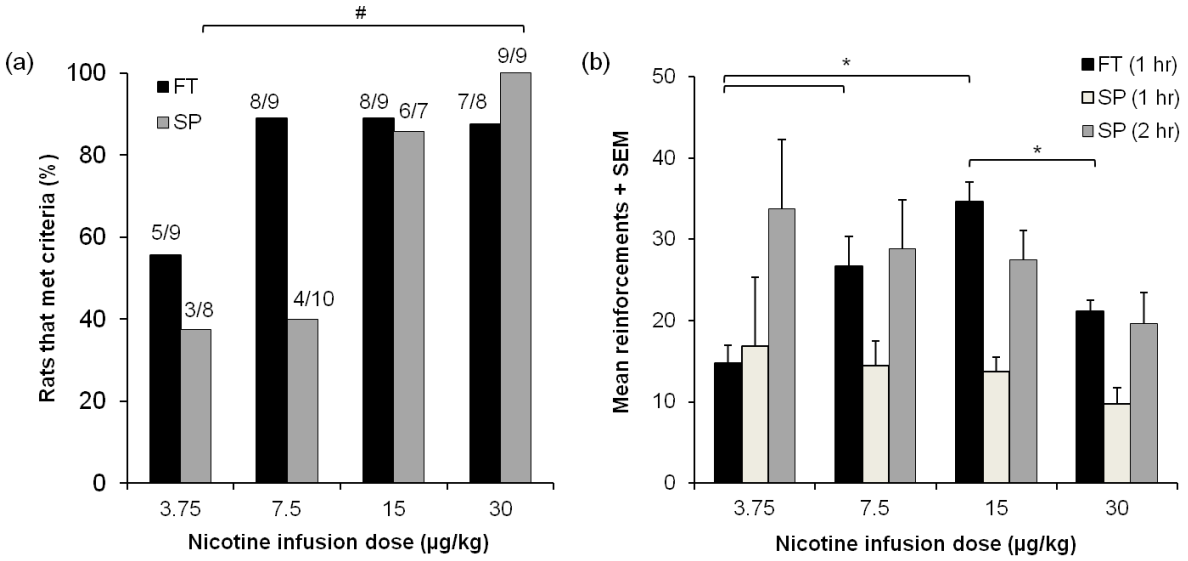

Figure 7. Acquisition of NSA is dependent on both prior food training and training dose but once animals have acquired, NSA behavior is dependent on training dose alone. **(a)** In FT groups, animals acquired NSA (approximately 90%) for all nicotine infusion doses except for the 3.75 µg/kg dose. In SP groups, animals acquired NSA dose-dependently with a greater proportion of animals acquiring with increasing infusion dose and shifted the dose-response curve for nicotine to the right compared to FT. Fractions at top of each bar indicate the number of animals that acquired compared to total number of animals. **(b)** Mean reinforcements were

dose-dependent for FT rats but there was no significant difference for SP rats. NSA data is from the mean of the last 3 sessions of the FR2 schedule. $p < 0.05$ denoted for FT (*) and SP (#). SEM = standard error of the mean.

1.2 Motivation to respond for nicotine measured by the PR schedule of reinforcement was greater with prior food training compared to no prior food training

FT and SP animals also underwent the PR schedule, where the breakpoint (final completed ratio of responding) was the endpoint measure. FT and SP rats that acquired NSA underwent PR at three different nicotine infusion doses (7.5, 15, and 30 µg/kg). FT animals had higher mean final completed ratios compared to SP at 7.5 µg/kg (31 ± 11 (SEM) vs. 9 ± 7), 15 µg/kg (121 ± 30 vs. 50 ± 17), and 30 µg/kg (94 ± 16 vs. 54 ± 6) ($F[1, 36] = 9.155$, $p = 0.005$), where a significant difference was found between FT and SP at 30 µg/kg ($p = 0.019$) (Figure 8). Higher final completed ratios is thought to reflect greater motivation because more work (responding) is required to receive one infusion of nicotine; therefore, this suggests that prior food training increased the motivation to obtain nicotine compared to spontaneous acquisition.

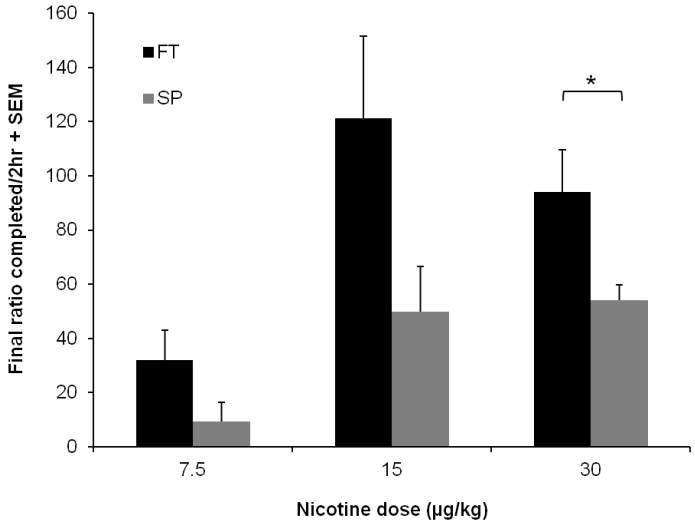

Figure 8. FT animals reached higher final completed ratios compared to SP animals responding under a PR schedule. Mean values for each nicotine infusion dose group were calculated from

mean data of the last 3 sessions within animal. *p<0.05, using log transformed data between FT and SP. SEM = standard error of the mean.

1.3 Head-to-head comparison of nicotine self-administration in animals with and without prior food training

The data for FT vs. SP from Section 1.1 and 1.2 was collected from two separate animal cohorts under different study conditions, which made the behavioral observations difficult to directly compare if animal shipment, supply of nicotine, or another factor related to animal cohort influenced the findings. Therefore in order to confirm that these differences in acquisition and PR were due to our independent variable (prior food training), a direct comparison of nicotine self-administration in rats food trained vs. not food trained was conducted. This comparison was examined using an infusion dose of nicotine (7.5 µg/kg) where there was a significant effect of food training vs. spontaneous training on acquisition (Section 1.1). In the following experiments, animals that food trained or did not have prior food training underwent acquisition, self-administration under an FR and PR schedule, and finally extinction and reinstatement of nicotine-seeking behaviour.

1.3.1 Prior food training increased proportion of rats that acquired nicotine self-administration

The proportion of rats that met acquisition criteria with SP (57 %) was significantly lower than the proportion of rats that met acquisition criteria with FT (100 %) (Figure 9a, Fisher's Exact, p = 0.002), replicating and extending the findings from Section 1.1. This suggests that food training can facilitate NSA acquisition at one of the lower nicotine infusion doses in the absence of other NSA parameters differences such as session duration (1 hour vs. 2 hour) and access (intermittent vs. continuous daily sessions). In the cue only condition, no FT animals and one of twelve SP animals met acquisition criteria, demonstrating that the higher proportion of rats that acquired NSA with FT was not due to a facilitative effect of FT on the secondary cues (light and tone) associated with NSA (Figure 9a).

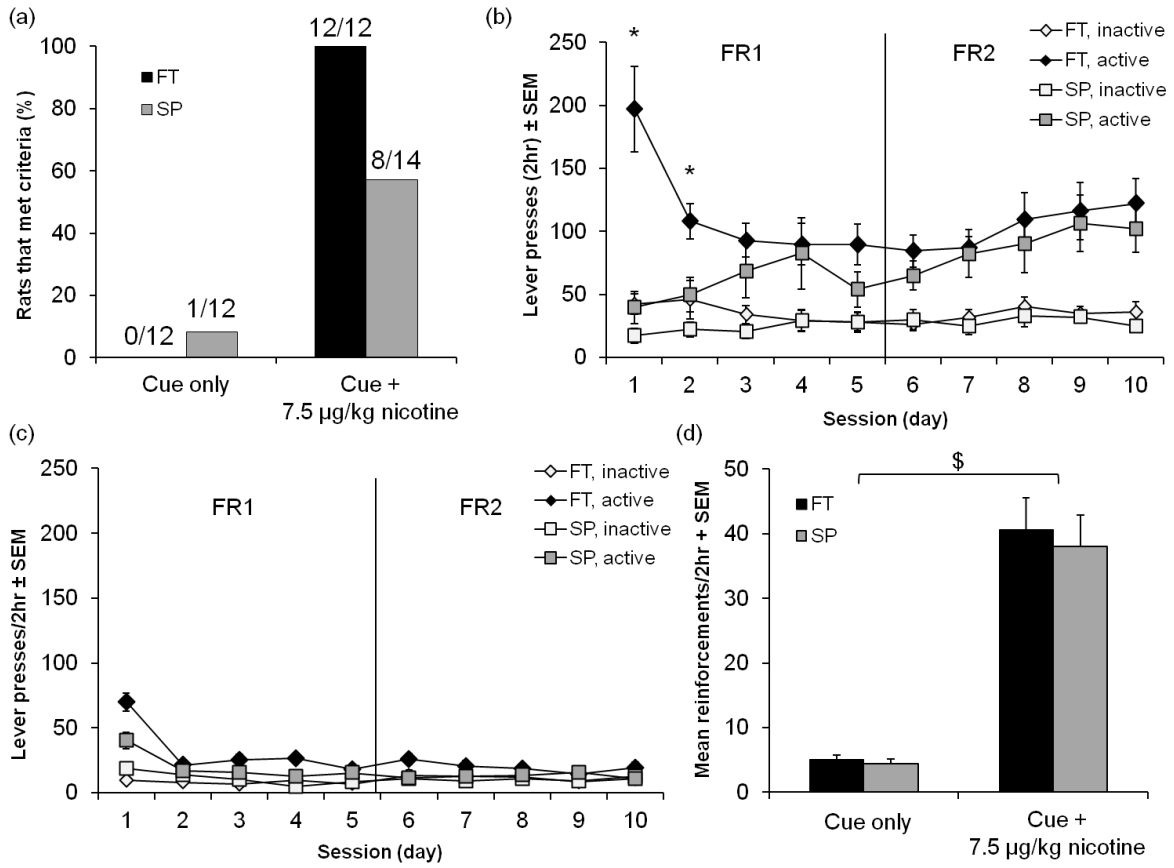

Figure 9. A greater proportion of FT animals acquired NSA compared to SP animals, but after acquisition FT animals did not respond differently from SP animals. **(a)** All FT animals acquired NSA compared to approximately 60% of SP animals. **(b)** Mean active and inactive lever presses by session in FT and SP rats (acquired animals; FT [n=12] and SP [n=8]). Active lever presses were different between FT and SP with nicotine reinforcement in the first 2 sessions. **(c)** Mean active and inactive lever presses by session for FT and SP rats that underwent cue only SA (all animals). Active lever presses were not different under the cue only condition. **(d)** Mean nicotine reinforcements between both FT and SP animals were not different in both cue and nicotine SA conditions. Mean reinforcements between cue only and nicotine SA were significantly different. *$p < 0.05$, FT vs. SP, \$$p < 0.001$, cue vs. nicotine. SEM = standard error of the mean.

1.3.2 Prior food training did not influence FR responding after acquisition

In animals that received cue plus nicotine infusions, data analysis of nicotine self-administration behaviour was conducted using only the animals that acquired. In the cue without nicotine condition, only one animal in SP met acquisition criteria, so for this cue only condition mean reinforcements for all FT and SP animals were included in analyses. In animals that received nicotine infusions, active lever presses were significantly higher in FT compared to SP rats ($F[1,18] = 5.154$, $p = 0.036$) in the first ($p = 0.004$) and second ($p = 0.011$) sessions during FR1 (Figure 9b). However, there was no significant difference in active lever presses, mean nicotine reinforcements or total nicotine intake between FT and SP rats by the end of FR2 (Figure 9d). Greater active lever presses within the first two FR1 sessions are consistent with greater acquisition in the FT group compared to SP. All FT rats met acquisition criteria while only a portion (8 out of 14) of SP animals met criteria; therefore the data presented in the SP group is averaged from animals that met criteria. The six animals that were excluded did not meet criteria mainly because responding on both active and inactive levers was the same (mean = 45 ± 20 (SEM) for both active and inactive lever presses during FR2), which violates the 2:1 preference for the active lever criterion. In addition, four of these animals displayed low and decreasing active lever presses as sessions continued (data not shown). In the cue only condition, there was no difference between FT and SP responding in FR1 or FR2 (Figure 9c). Mean reinforcements in response for the cue alone was not significantly different ($F[1,22] = 0.79$, $p = 0.38$ for FR1 and $F[1,22] = 2.09$, $p = 0.16$ for FR2) between FT and SP rats (Figure 9d). These results demonstrate that prior food training did not increase responding for the secondary cues in the absence of nicotine and provides additional support that prior food training facilitates responding for nicotine infusions.

1.3.3 Prior food training did not influence PR responding after acquisition

FT and SP rats that acquired NSA underwent PR sessions after FR2 sessions were completed. The mean final completed ratio for FT and SP rats under PR at each nicotine dose are presented in Figure 10, where no difference between FT and SP animals ($p = 0.132$) was observed. There was a significant difference between doses ($F[2,28] = 35.411$, $p < 0.001$) where final completed ratios were significantly different between all doses regardless of whether animals were trained or not: 7.5 µg/kg vs. 15 µg/kg ($p = 0.027$), 7.5 µg/kg vs. 30 µg/kg ($p < 0.001$), and 15 µg/kg vs. 30 µg/kg ($p < 0.001$). Separate analysis of FT and SP animals show dose-dependent increases in

mean final completed ratios for FT animals ($F[2, 14] = 22.813$, $p < 0.001$) and SP animals ($F[2, 14] = 14.415$, $p < 0.001$) (Figure 10). Post-hoc pair-wise comparisons with Bonferroni correction show that in FT animals, mean final completed ratio between 7.5 µg/kg vs. 30 µg/kg and 15 µg/kg vs. 30 µg/kg were significantly different ($p < 0.001$ and $p = 0.004$, respectively). In SP animals, mean final completed ratios between 7.5 µg/kg vs. 15 µg/kg trended toward significance ($p = 0.060$) and were significantly different between 7.5 µg/kg vs. 30 µg/kg and 15 µg/kg vs. 30 µg/kg ($p = 0.015$ and $p = 0.019$, respectively). These results suggest that food training does not influence motivation to respond for nicotine and that the effect seen in Section 1.2 with separate FT and SP cohorts could be due to the influence of other NSA parameters on motivation, such as FR session duration prior to PR (1 hour vs. 2 hour) and access schedule (intermittent vs. continuous daily sessions).

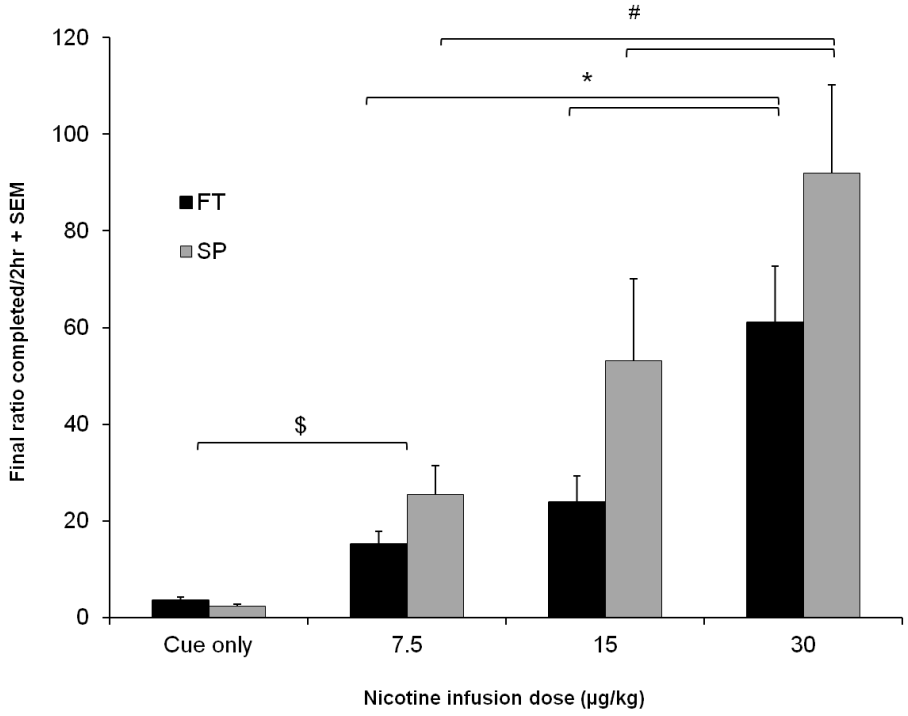

Figure 10. Final ratios completed under the PR schedule were not different between FT and SP animals. This was observed in both cue only (all animals [n=12]) and nicotine infusion self-administration conditions (shown for acquired animals; FT and SP [n=8]). Within training paradigm, significant dose-dependent differences in final ratio completed were found. Mean

values for each dose group were calculated using mean values of the last 3 sessions for each animal. Mean inactive lever presses (± SEM) for the last 3 sessions in FT animals were 15 ± 3 for 7.5 µg/kg, 16 ±5 for 15 µg/kg, and 41 ± 10 for 30 µg/kg; in SP animals they were 19 ± 7 for 7.5 µg/kg, 30 ± 10 for 15 µg/kg, and 48 ± 12 for 30 µg/kg. $p<0.05$ for nicotine doses within FT (*) and SP (#) groups, $p < 0.001$ between cue only vs. nicotine SA ($). SEM = standard error of the mean.

Mean final completed ratios for the cue only condition include data from all animals. There was little responding for cue alone in either FT or SP animals (Figure 10) where the mean final completed ratio completed was 3 for FT and 2 for SP animals. In comparison, the mean final completed ratio at the lowest nicotine infusion dose (7.5 µg/kg) was significantly higher than the cue only condition (15 for FT and 25 for SP animals; cue vs. nicotine, $F[1,39] = 51.7$, $p < 0.001$). These results demonstrate that responding in the nicotine + cue condition under these NSA parameters was primarily due to the presence of nicotine during PR and that the cues alone do not elicit responding to the same magnitude as that with nicotine.

1.3.4 Prior food training did not influence nicotine-seeking behavior

There was no difference in responding during extinction between SP and FT rats (Figure 11a). Following session 4 (Figure 11a), rats began to meet extinction criteria and were subsequently tested for reinstatement. In extinction, there was a significant effect of session ($F[1.6, 26.5] = 25.296$, $p<0.001$), as responding decreased over days, but there was neither a significant effect of training ($F[1, 17] = 1.15$, $p = 0.3$) nor was there an interaction between session x training ($F[1.6, 26.5] = 3.467$, $p = 0.056$). Also, the number of sessions required to reach extinction criteria was analyzed between FT and SP rats. FT rats averaged 10 ± 1 sessions while SP rats averaged 14 ± 3 sessions; however, this was not significant ($t[9.533] = -1.155$, $p = 0.276$).

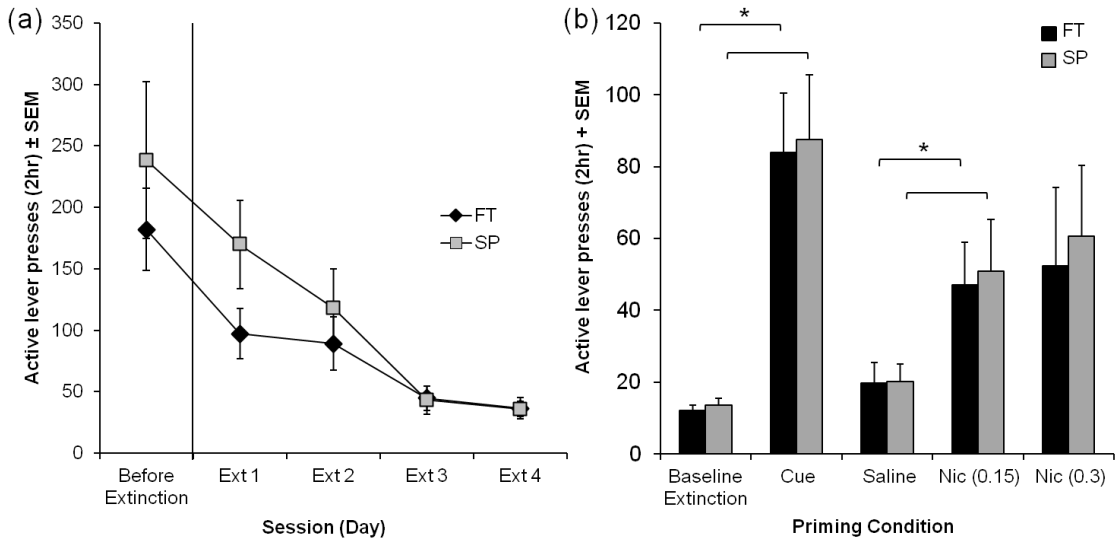

Figure 11. There was no difference in extinction and reinstatement between FT and SP animals (n=8 per group). **(a)** Mean active lever presses during extinction sessions with no cues were similar between FT and SP animals. The first four extinction sessions are shown. Mean inactive lever presses (± SEM) for these four extinction sessions are 17 ± 5, 20 ± 6, 10 ± 3, 9 ± 3 for FT and 18 ± 5, 18 ± 4, 14 ± 5, 12 ± 5 for SP. **(b)** Mean active lever presses at baseline (the last two extinction sessions for each animal), and during the cue, saline, and two nicotine priming sessions are shown. Mean responding was similar for cue- and nicotine-induced reinstatement (Nic: 0.15 and 0.3 mg/kg/sc) in both training groups. Mean inactive lever presses (± SEM) in FT were 6 ± 2 for baseline, 11 ± 3 for cue, 8 ± 2 for saline, 10 ± 3 for Nic (0.15 mg/kg) and 18 ± 6 for Nic (0.3 mg/kg). Mean inactive lever presses (± SEM) in SP were 3 ± 1 for baseline, 8 ± 2 for cue, 5 ± 2 for saline, 22 ± 4 for Nic (0.15 mg/kg) and 20 ± 7 for Nic (0.3 mg/kg). $p<0.05$, cue vs. baseline extinction and saline vs. 0.15 mg/kg nicotine for FT (*) and SP (#) rats. SEM = standard error of the mean.

Both FT and SP rats reinstated nicotine-seeking behaviour when exposed to the compound cue previously associated with nicotine infusions (Figure 11b). Active lever presses in response to the compound cue were significantly higher than during extinction ($F[1,17] = 34.671$, $p < 0.001$). There was no difference between FT and SP rats in active lever presses during cue priming.

Animals re-commenced extinction sessions until they met extinction criteria and nicotine-induced reinstatement was tested. Responding after one saline SC injection, a baseline for nicotine priming injections, was not different between FT and SP animals. Responding after priming SC injections of either saline, 0.15 mg/kg nicotine, or 0.3 mg/kg nicotine was significantly different ($F[2, 34] = 3.54$, $p = 0.026$). Post-hoc pair-wise comparisons with Bonferroni correction showed that responding after the 0.15 mg/kg nicotine priming dose was significantly higher than responding after saline ($p = 0.02$). There was no difference in responding to either nicotine priming dose between FT and SP animals (Figure 11b). These results suggest that once animals have acquired NSA their responding and motivation to seek nicotine are reinforced by the nicotine infusion dose.

2 Effect of brain CYP2B inhibition on intravenous nicotine self-administration behaviour

The effect of inhibiting brain CYP2B on this nicotine self-administration was examined using the information on infusion dose and use of prior food training in Section 1. The activity of brain CYP2B was reduced by chemical inhibition: in these experiments rats were treated with the selective CYP2B inhibitor, C8X, and/or its vehicle, ACSF specifically in the brain via ICV injection at a dose previously established to inhibit CYP2B and alter the centrally-mediated behaviour of other CYP2B substrates such as propofol (Khokhar and Tyndale, 2012) and chlorpyrifos (Khokhar and Tyndale, 2013). C8X and ACSF ICV-treated rats then underwent the various stages of nicotine self-administration such as acquisition, motivation, extinction and reinstatement. Results are modified version of Garcia et al. (2015) (doi: 10.1038/npp.2015.40).

2.1 ICV injection of C8X increased acquisition of nicotine self-administration behavior but did not alter responding after acquisition

From Section 1.1, the dose range from 3.75 to 15 µg/kg yielded an ascending dose-response curve for acquisition where only a portion of animals acquired NSA at the lower doses. This was seen with vehicle ICV injections, where the proportion of rats that acquired nicotine self-

administration increased with increasing nicotine infusion doses from 3.75-15 µg/kg (Figure 12a). With 3.75 and 7.5 µg/kg training doses around 40% met acquisition criteria and with the 15 µg/kg training dose almost all rats (86%) met criteria. With C8X ICV injections, acquisition with the lowest infusion dose (3.75 µg/kg) was low, where approximately 20% of rats acquired the behaviour; however, at 7.5 µg/kg and at 15 µg/kg essentially all animals acquired (Figure 12a). In rats that self-administered 3.75 µg/kg, 3 out of 8 acquired with ACSF vs. 2 out of 9 acquired with C8X; for the 7.5 µg/kg dose 10 out of 22 acquired with ACSF vs. 21 out of 21 acquired with C8X; and for the 15 µg/kg dose 6 out of 7 acquired with ACSF vs. 7 out of 9 with C8X. Data for 7.5 µg/kg dose was combined from two animal cohorts: In the first cohort 4 out of 10 animals acquired with ACSF vs. 11 out of 11 acquired with C8X and in the second cohort 6 out of 12 animals acquired ACSF vs. 10 out of 10 acquired with C8X. Combined, this increase in the proportion of rats that acquired self-administration at the 7.5 µg/kg with C8X ICV treatment compared to vehicle was significantly different ($p < 0.001$, Fisher's Exact), suggesting a leftward shift in the dose-response for acquisition. Greater acquisition at lower doses with inhibitor pre-treatment suggests that reducing brain CYP2B activity increased the apparent dose of nicotine. As the infusion doses tested were on the ascending limb of nicotine's dose-response curve, greater apparent doses would increase the reinforcing effect of nicotine.

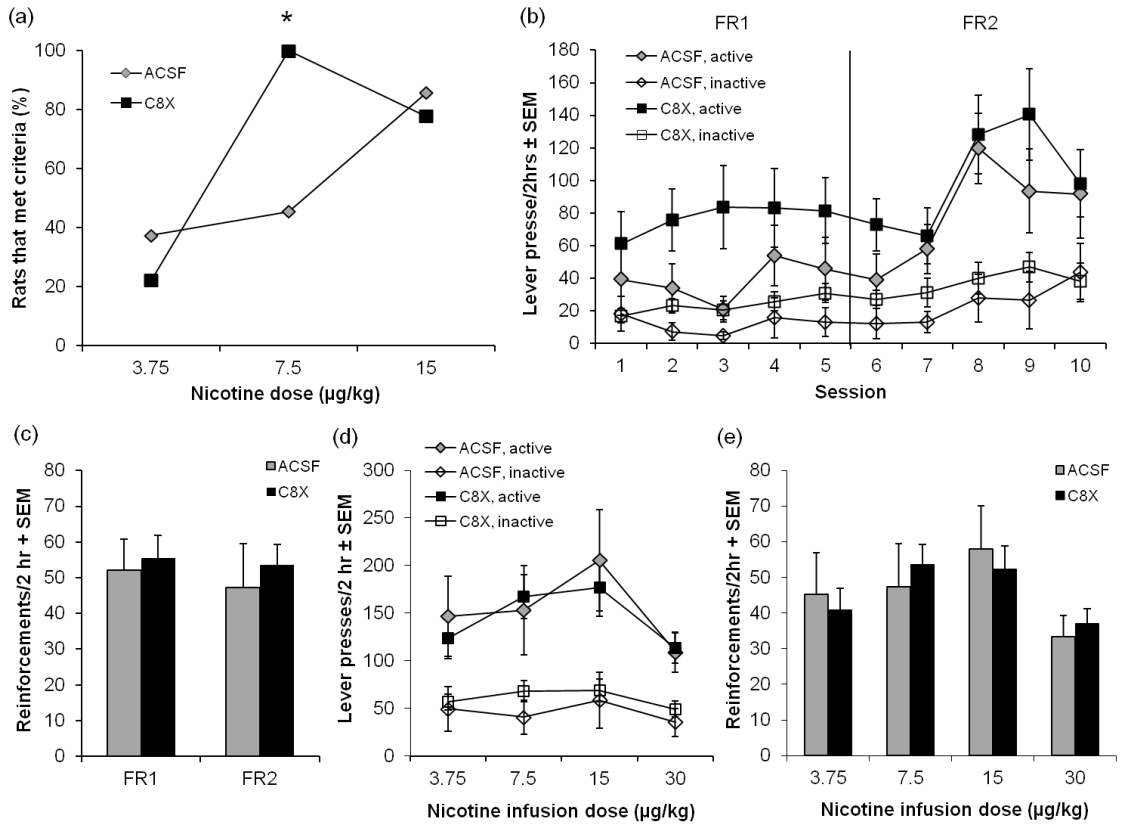

Figure 12. ICV injection of C8X results in a leftward shift of the acquisition dose-response for nicotine but does not alter NSA after behaviour is acquired. **(a)** The proportion of rats that acquired NSA with C8X was significantly higher compared to ACSF at the 7.5 µg/kg infusion dose when data from two cohorts was analyzed separately ($p < 0.05$ for each cohort) or together. * $p < 0.001$, C8X vs. ACSF. FR1 and FR2 **(b)** mean active and inactive lever presses by session and **(c)** mean reinforcements were not different between C8X and ACSF-treated rats that acquired at the 7.5 µg infusion dose. **(d)** Active lever presses and **(e)** mean reinforcement dose-response curves under an FR2 schedule were also not different between C8X and ACSF. Data in c, d, and e are from last 3 NSA sessions. SEM = standard error of the mean.

The effect of C8X ICV treatment on responding during nicotine self-administration was examined, where mean active lever presses and reinforcements earned under FR1 and FR2 were compared in rats that met acquisition criteria. This was examined in the groups that trained at the 7.5 µg/kg dose of nicotine where there was a significant effect of C8X treatment on acquisition.

There was no difference in mean active and inactive lever presses by session (Figure 12b) nor in mean reinforcements (Figure 12c) during FR1 and FR2 between C8X and ACSF-treated rats. Analysis excluded rats that did not meet acquisition criteria, which were all ACSF-treated. These rats displayed similar mean active and inactive lever presses (FR1: 14 ± 6 (SEM) vs. 10 ± 6, FR2: 8 ± 2 vs. 9 ± 6) and reinforcements dropped from FR1 to FR2 (14 ± 7 to 9 ± 6).

To determine if C8X ICV treatment could alter responding in these animals when the infusion dose was changed, rats were tested at different nicotine infusion doses under the FR2 schedule. Mean active, inactive lever presses, and reinforcements for each infusion dose were not different between ICV treatments at all nicotine doses (Figures 12d and 12e), suggesting that once animals acquired the behaviour their responding was driven by the nicotine infusion dose. This suggests that once a stable pattern of behaviour is established the contribution of brain CYP2B activity may be superseded by systemic levels of nicotine.

2.2 ICV injection of C8X increased the motivation to respond for nicotine on a PR schedule of reinforcement

To test the effect of C8X on the motivation to obtain nicotine rats underwent FR1 and FR2 and PR training without exposure to C8X. Rats underwent five PR sessions to establish a baseline level of responding. They were then treated with ACSF and tested under PR. There was no difference in mean final completed ratio between baseline PR and ACSF treatment which indicated no significant effect of ICV injection on PR responding (data not shown). After ACSF ICV PR sessions, rats were then given C8X ICV and tested under PR. The mean final completed ratio for rats after C8X treatment were higher compared to their ACSF treatment at all infusion doses (Figure 13). Comparing ICV treatment within dose group revealed that this was significantly different at 0.015 g/kg [$F(1,7) = 7.365$, $p = 0.03$]. These results suggest that inhibiting brain CYP2B activity by C8X increased the motivation to respond for nicotine, possibly by increasing the apparent dose of each nicotine infusion received.

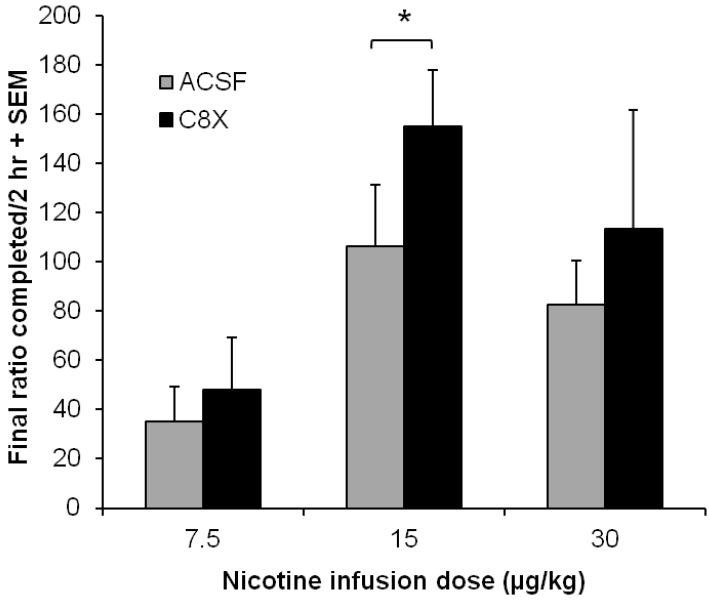

Figure 13. ICV injection of C8X can increase motivation to obtain nicotine in the PR schedule. C8X increased final ratios completed during PR compared to ACSF for animals trained at 7.5 µg/kg (n=8), 15 µg/kg, (n=8), and 30 µg/kg (n=7). Mean final ratio completed was calculated using the mean log transformed final ratio from the 3 sessions following ICV injection of ACSF or C8X. *$p < 0.05$, C8X vs. ACSF. SEM = Standard error of the mean.

2.3 ICV injection of C8X increased the persistence to respond for nicotine during extinction but did not alter nicotine-seeking behavior during reinstatement

In the four daily extinction sessions active lever presses between rats treated with C8X compared to ACSF were not significantly different (Figure 14a). In addition to the four daily extinction sessions, animals underwent multiple extinction sessions until they met extinction criteria on the same day reinstatement tests were conducted. The number of sessions animals needed before they met extinction criteria was compared between C8X and ACSF-treated rats, where C8X animals on average required more sessions to meet criteria compared to ACSF (Figure 14b, Kaplan-Meier survival analysis using log-rank test, $\chi^2=4.65$, $p = 0.03$).

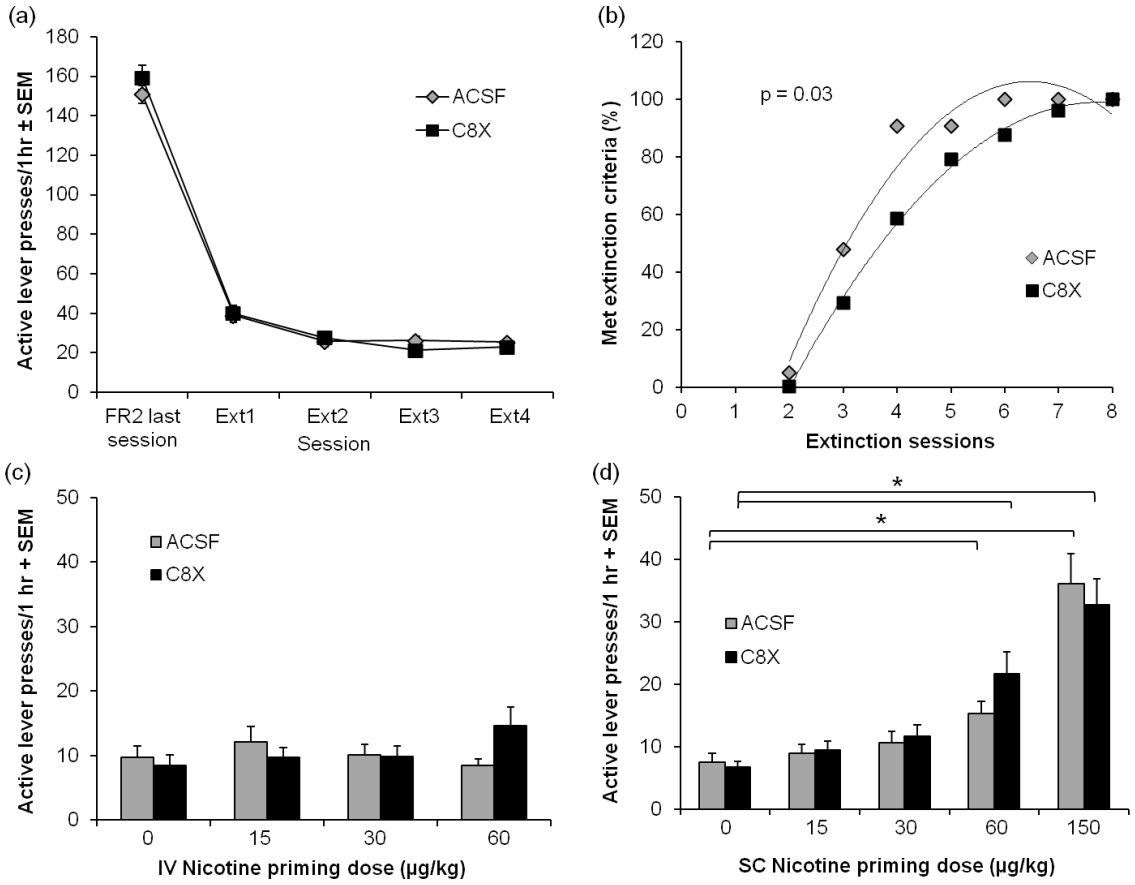

Figure 14. ICV injection of C8X can increase motivation to obtain nicotine in nicotine-seeking behaviour. **(a)** Mean active lever presses (± SEM) for the first four daily extinction sessions (C8X: 40 ± 6, 28 ± 4, 21 ± 2, and 23 ± 3; ACSF: 39 ± 4, 26 ± 4, 26 ± 3, and 25 ± 2) were not different between C8X (n=24) and ACSF (n=21) groups. **(b)** Animals then underwent multiple extinction sessions the same day until they reached extinction criteria. Data shown is the proportion of C8X and ACSF rats that met extinction criteria over these extinction sessions and C8X-treated animals needed more sessions to meet criteria. During nicotine reinstatement testing mean active lever presses between C8X and ACSF were not different after **(c)** IV (C8X: n=17, ACSF: n=18) or **(d)** SC (C8X: n=24, ACSF: n=21) nicotine priming injections at all doses tested. Mean active lever presses between C8X and ACSF after 60 µg/kg/IV approached significance ($p = 0.06$). *$p < 0.05$, saline (0 µg/kg nicotine) vs. 60 and 150 µg/kg/SC for both C8X and ACSF. SEM = standard error of the mean.

Once animals met extinction criteria, rats were given one saline injection (IV or SC) and underwent one session to determine a baseline level of responding. Active lever presses during extinction were not different compared to active lever presses after the saline injection (data not shown). Animals were then tested for reinstatement in subsequent sessions with increasing nicotine priming doses given IV (0.015, 0.03. 0.06 mg/kg). In both ICV treatments, rats did not reinstate their nicotine-seeking behaviour at any nicotine priming dose tested because active lever presses at all nicotine priming doses were not significantly different from saline (Figure 14c). When active lever presses between C8X and ACSF groups were compared, there was no significant difference at any of the nicotine priming doses. After this reinstatement test day, animals returned to extinction and were tested with nicotine priming doses given SC (0.015, 0.03, 0.06, 0.15 mg/kg). Within each ICV treatment group there was a significant effect of priming dose on active lever presses for C8X [$F(4, 88) = 22.403$, $p < 0.001$] and ACSF [$F(2.735, 51.985) = 15.669$, $p < 0.001$], where rats reinstated their nicotine-seeking behavior after receiving 0.06 (C8X, $p < 0.001$; ACSF, $p = 0.003$) and 0.15 mg/kg (C8X, $p < 0.001$; ACSF, $p < 0.001$) priming doses given SC. When active lever presses were compared between C8X and ACSF groups, there was no significant difference (Figure 14d). The relative persistence in extinction with C8X vs. ACSF ICV treatment suggests that lower brain CYP2B activity may increase the motivation to respond for nicotine. Greater mean responding during IV and SC reinstatement in C8X-treated rats compared to ACSF trended toward significance, consistent with increased motivation for nicotine because rats were willing to respond more after receiving the priming injection of nicotine. This suggests that with C8X ICV treatment, the association of nicotine with responding on the active lever was stronger than ACSF ICV treatment.

2.4 ICV injection of C8X did not alter peripheral levels of nicotine and its metabolite cotinine

To confirm that the dose of C8X injected ICV during NSA did not inhibit nicotine metabolism in the liver, plasma levels of nicotine and cotinine were measured at the end of behavior (Figure 15). The levels of plasma nicotine and cotinine collected after an IV bolus injection of nicotine (0.2 mg/kg) were not different in animals that were given ICV injections during PR in Section 2.2 (Figure 15a). The levels of plasma nicotine and cotinine collected after four 7.5 μg/kg

infusions of nicotine were also not different in animals that were given ICV injections during acquisition in Section 2.1 (Figure 15b). These results demonstrate that repeated ICV administration of the inhibitor did not alter peripheral nicotine metabolism.

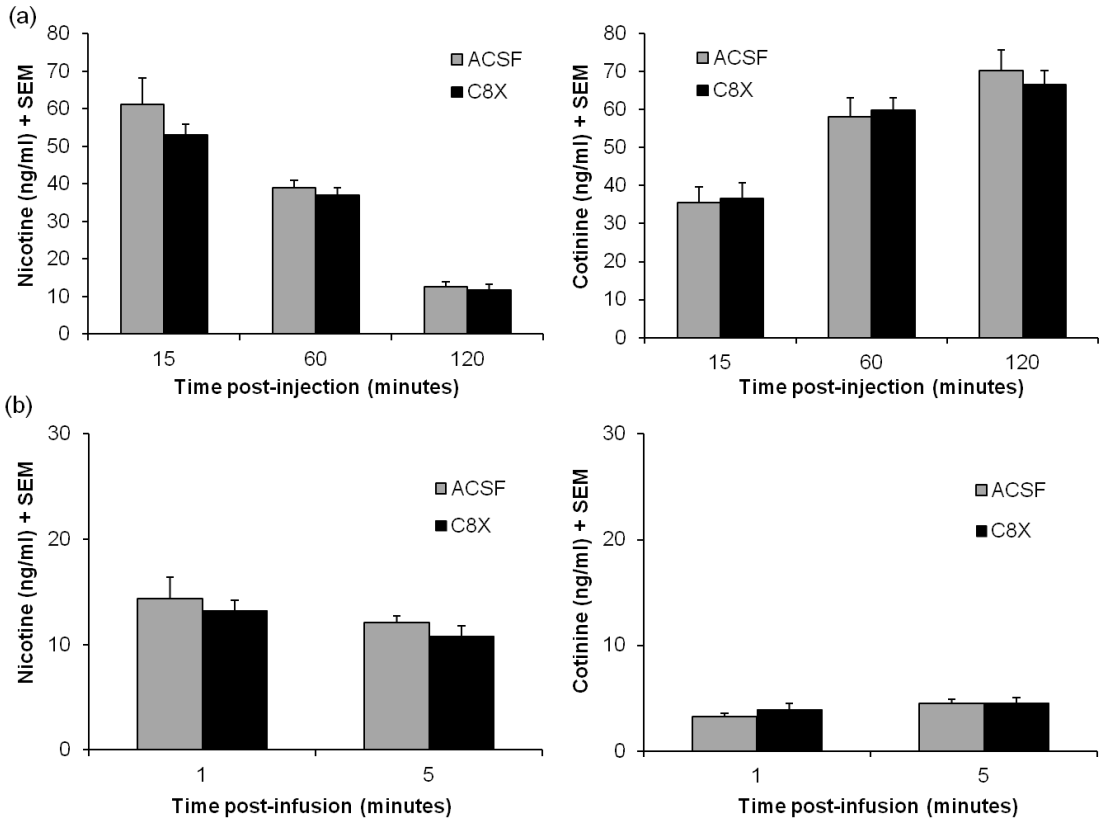

Figure 15. ICV injection of C8X does not alter peripheral nicotine metabolism in animals that underwent NSA. In rats treated with C8X or ACSF plasma nicotine and cotinine levels were measured after **(a)** a bolus IV injection of nicotine (0.2 mg/kg) (ACSF [n=12]; C8X [n=13]), and **(b)** Four nicotine infusions given in the operant SA chamber (7.5 μg/kg/infusion) (ACSF [n=8]; C8X [n=8]) at the end of NSA. In both nicotine challenges, neither nicotine nor cotinine plasma levels differed between C8X and ACSF groups. SEM = standard error of the mean.

3 Effect of brain CYP2B inhibition and induction on nicotine brain levels measured by *in vivo* microdialysis

ICV injections of the CYP2B inhibitor C8X were able to alter NSA behaviour where C8X treatment increased NSA acquisition and motivation to respond for nicotine, suggesting that altering brain CYP2B could influence nicotine reinforced behaviour. The next question was whether altering brain CYP2B could influence brain levels of nicotine. ICV injections of C8X at this same inhibitor dose can alter the brain levels of two other CYP2B substrates, propofol (Khokhar and Tyndale, 2011) and chlorpyrifos (Khokhar and Tyndale, 2012). Brain propofol levels (the active compound) were elevated and chlorpyrifos-oxon levels (the active metabolite) were lower while plasma levels of the parent and metabolite were unaltered by the ICV inhibitor treatment suggesting that ICV inhibitor treatment reduced brain but not hepatic CYP2B activity.

Propofol and chlorpyrifos-oxon levels in the brain were measured in whole brain homogenates at a single point in time after drug administration, following sacrifice of the animals. If inhibiting CYP2B activity can alter brain levels of its substrates at one time point, then measuring this effect over time can show how inhibition alters the substrate's pharmacokinetics, specifically the time course of this effect on brain nicotine when given peripherally. This can be tested using *in vivo* microdialysis, where the drug can be repeatedly sampled in brain extracellular fluid. Therefore, to test whether ICV injection of C8X can increase and/or prolong nicotine levels in brain, nicotine microdialysis was carried out in rats pre-treated with C8X. Among the three brain microdialysis studies published for nicotine (IV: Chang et al., 2005; Woods et al., 2006; SC: Katner et al., 2014), only one study examined nicotine brain levels following more than one dose of nicotine (Katner et al., 2014). Before testing the effect of the CYP2B inhibitor on brain nicotine levels in microdialysis, the dose range that would be within the limits of detection for quantification, and that could elicit increases in brain nicotine levels with increasing peripheral nicotine dose was determined. Once the nicotine microdialysis protocol was established, brain nicotine levels were measured from brain dialysate collected after a single bolus IV injection of nicotine in animals treated ICV with inhibitor or vehicle. Results modified version of Garcia et al. (2015) (doi: 10.1038/npp.2015.40).

3.1 Effect of brain CYP2B inhibition on brain nicotine levels

3.1.1 Nicotine brain levels following a single IV injection of nicotine increase dose-dependently

Brain nicotine levels measured in the right lateral ventricle after IV injection of 0.15, 0.225, or 0.30 mg/kg increased within the first 30 minutes post-injection (Figure 16a), where a dose-dependent increase can be seen at the 15-30 minute collection time point, which also corresponds to the peak concentration for nicotine (Figure 16b). After 30 minutes brain nicotine levels decreased to similar levels regardless of dose (Figure 16a). The lowest nicotine dose (0.15 mg/kg) resulted in a mean peak nicotine concentration of 4.6 ng/ml; therefore, because the limit of detection for the LC-MS nicotine assay is 1 ng/ml, this was the lowest dose of nicotine used for nicotine microdialysis because a lower dose of nicotine would yield peak nicotine levels that were potentially below the limit of detection. Plasma levels of nicotine collected at 15 and 75 minutes post-injection also increased dose-dependently (Figure 16c, $F[2, 12] = 10.41$, $p = 0.02$). This shows that increasing IV nicotine doses resulted in increasing nicotine plasma levels.

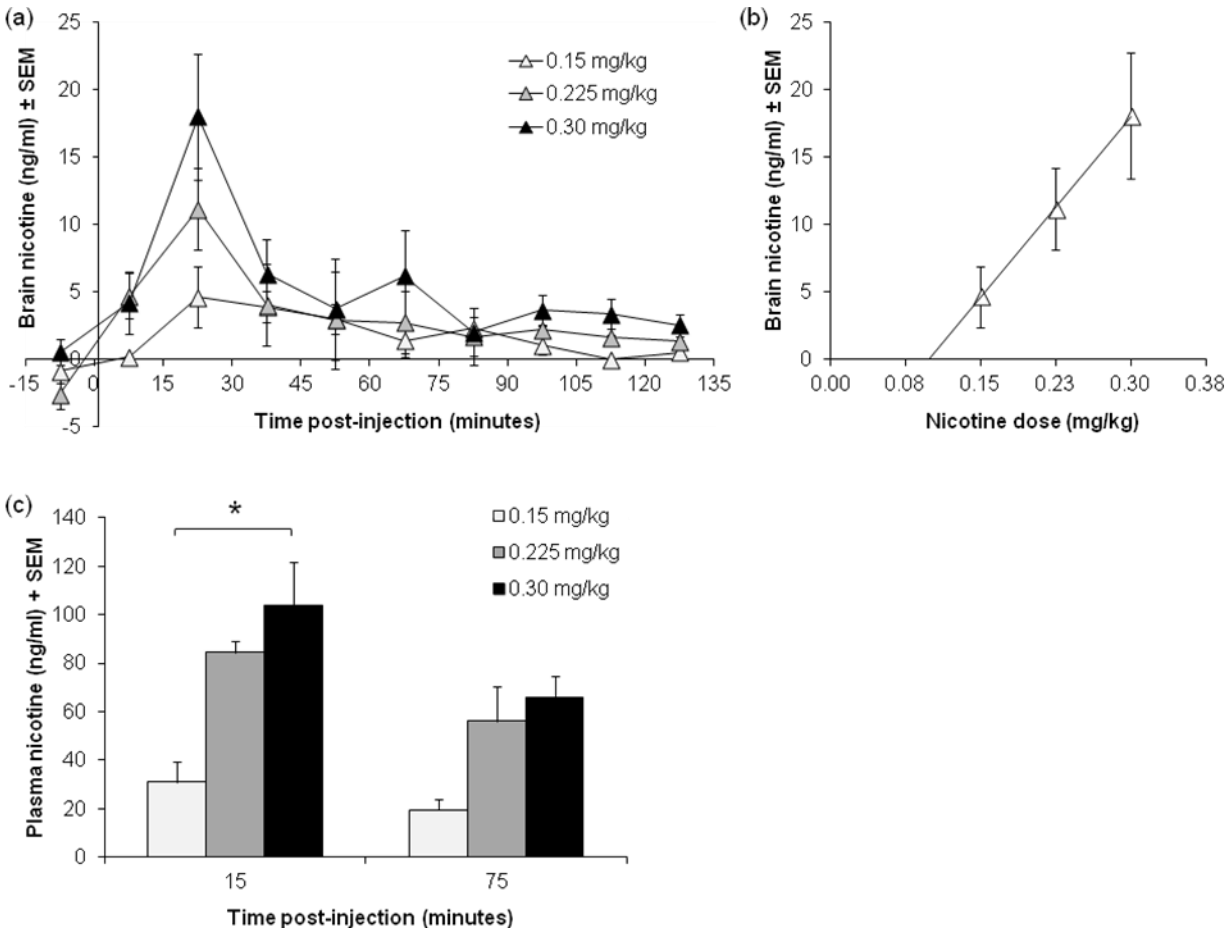

Figure 16. Brain nicotine levels measured by *in vivo* microdialysis increase dose-dependently after an IV injection of nicotine. **(a)** Nicotine brain levels over time in animals given a single IV injection of nicotine. Three different doses were used, 0.15, 0.225, and 0.30 mg/kg (n=5/dose). **(b)** Nicotine brain levels at 15-30 minutes post-injection show a dose-dependent increase. **(c)** Plasma nicotine levels dose-dependently increased at 15 minute post-nicotine. *$p < 0.05$. SEM = standard error of the mean

3.1.2 ICV injection of C8X increased nicotine brain levels within first 45 minutes after a single IV injection of nicotine

If C8X ICV injection inhibits CYP2B activity, then the brain nicotine levels are expected to be higher than with ASCF ICV. Therefore, for nicotine microdialysis where animals were given inhibitor or vehicle, the lowest dose of nicotine where brain levels are above the limit of

detection was used, which was 0.15 mg/kg. After a single IV injection of nicotine, nicotine brain levels in the right lateral ventricle were significantly higher at the 15-30 minute collection time point ($t[17]= -2.27$, $p=0.04$) with C8X treatment compared to ACSF-treated rats (Figure 17a). Nicotine brain levels were also significantly higher throughout the first 45 minutes post-injection in C8X-treated rats compared to ACSF ($t[17]=2.23$, $p=0.04$), but were not different at subsequent time points (Figure 17b). This demonstrates that ICV injection of C8X can increase brain nicotine levels and suggests that lower brain CYP2B activity may result in less metabolism of nicotine. Plasma nicotine and cotinine levels were not different between C8X and ACSF-treated rats, suggesting that ICV injection of the inhibitor did not alter peripheral nicotine metabolism (Figure 17c).

Brain levels of the major metabolite cotinine were quantified, however cotinine levels were variable and low (< 3 ng/ml) and there was no difference between ACSF and C8X ICV treatment over time (data not shown). Other nicotine metabolites including nornicotine, 3-hydroxycotinine, norcotinine, and nicotine-N-oxide were also assessed however they were all below the limit of detection (1 ng/ml).

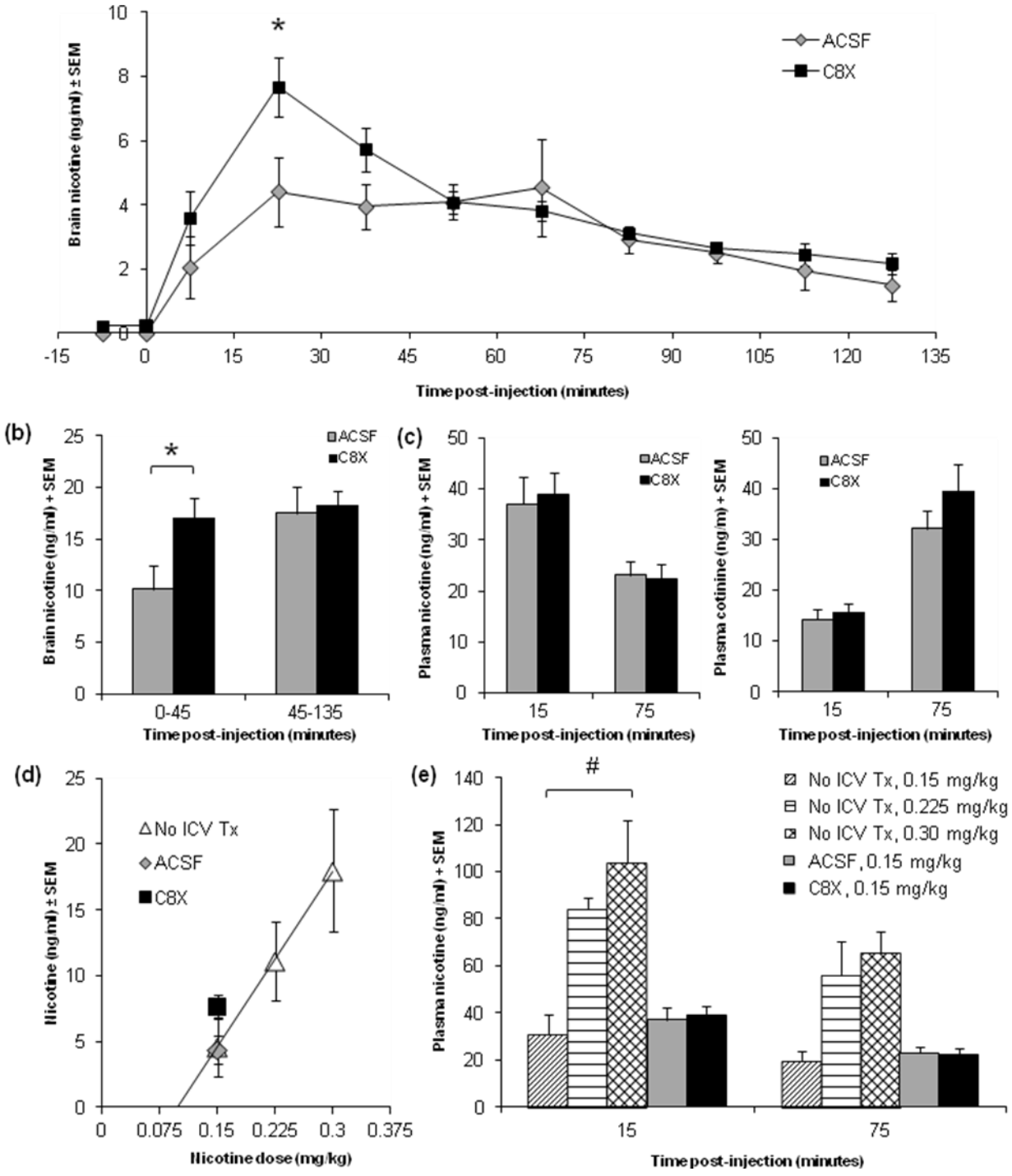

Figure 17. ICV injection of C8X increases circulating nicotine levels in the lateral ventricles measured by *in vivo* microdialysis. **(a)** Nicotine dialysate levels were higher after C8X treatment (n=9) compared to ACSF (n=10) 15-30 minutes post-injection (150 µg/kg/IV, which was the lowest dose of nicotine that produced brain dialysate concentrations of nicotine above the limit

of detection for our LC-MS assay [1 ng/ml]). **(b)** Total nicotine dialysate within 0-45 minutes post-injection were higher after C8X treatment compared to ACSF. **(c)** Nicotine and cotinine plasma levels obtained during microdialysis were not different between C8X and ACSF treatment. **(d)** Mean nicotine dialysate collected 15-30 minutes post-nicotine from C8X and ACSF-treated animals are shown in comparison to the nicotine dose-response curve for dialysate collected from No ICV Tx animals (n=5/dose). **(e)** Mean plasma nicotine levels for C8X and ACSF-treated animals are shown in comparison to the nicotine dose-response curve for plasma collected from No ICV Tx animals. SEM = standard error of the mean. *$p < 0.05$, C8X vs. ACSF, # $p < 0.05$, between doses within No ICV Tx

The nicotine brain levels after C8X and ACSF were compared to the dose-response curve generated in Section 3.1.1 above to provide an estimate of the equivalent change in peripheral nicotine dosing with C8X treatment (Dose-response labeled "No ICV Tx" in Figure 17d). Nicotine brain levels were similar to nicotine levels without ICV injection (No ICV) in animals given ACSF ICV, demonstrating that vehicle injections did not influence nicotine levels from microdialysis collection. In animals given C8X ICV (tested at 0.15 mg/kg nicotine) nicotine brain levels were higher than both No ICV Tx and ACSF groups, corresponding to a 25% increase in the apparent dose of peripherally administered nicotine. Plasma nicotine levels in ACSF and C8X-treated animals were similar to the levels measured in No ICV Tx animals (0.15 mg/kg, Figure 17e). These results indicate that ICV injections of C8X can increase nicotine levels in the brain, while leaving the systemic nicotine levels unchanged.

3.2 Effect of brain CYP2B induction on brain nicotine levels

3.2.1 Nicotine brain levels following a single SC injection of nicotine increase dose-dependently

The role of brain CYP2B activity on smoking behaviour was largely focused on inhibition in order to investigate whether reducing activity specifically in the brain would increase nicotine reinforced behaviour, which would support the theory that reduced brain CYP2B activity in human *CYP2B6* slow metabolizers could be playing a role in smoking behaviour. Greater brain

CYP2B activity is also possible, as many drugs that can cross the blood brain barrier are CYP2B inducers (Wang and Negishi, 2003; Faucette et al., 2004; Ferguson et al., 2013). If reduced CYP2B activity in the brain can increase nicotine brain levels than it is possible that increased CYP2B activity in the brain can decrease nicotine levels.

CYP2B can be induced in the brain, without induction in the liver, by 7 days of nicotine treatment (1 mg/kg/SC given once a day). This paradigm has been well established in previous studies from our lab where the protein levels of CYP2B in the brain, but not in the liver, increased following 7-day nicotine treatment (Miksys et al., 2000; Khokhar et al., 2010). The induction of CYP2B in the brain was sufficient to decrease propofol response, and thus is likely to be meaningful (Khokhar, 2011). In order to investigate if 7-day nicotine treatment can induce brain CYP2B resulting in reduced nicotine brain levels, animals underwent microdialysis before and after 7-day nicotine treatment in a within-animal design. As 7-day nicotine treatment was delivered SC (the route of administration in previous induction studies), and in order to keep exposure to nicotine consistent within each animal, nicotine microdialysis was conducted following a SC injection of nicotine at the same dose (1 mg/kg). To confirm that the effect of 7-day nicotine treatment was due to CYP2B induction in the brain, the CYP2B inhibitor and/or its ACSF vehicle was given before, during, or after 7-day nicotine treatment.

Brain nicotine levels of the 1.0 mg/kg/SC nicotine dose, measured over 75 minutes post-injection in the right lateral ventricle, were well above the limits of detection (Figure 18). Mean brain nicotine levels peaked to 25.6 ng/ml at 30-45 minutes post-injection (Figure 18a). Two lower nicotine doses, 0.6 mg/kg and 0.3 mg/kg, were also tested to demonstrate that brain nicotine levels measured by microdialysis were consistent with the peripheral nicotine dose. The 0.6 mg/kg nicotine dose yielded quantifiable nicotine levels, where brain levels of 14.8 ng/ml peaked at 15-30 minutes post-injection (Figure 18a). Brain nicotine levels at 15-30 minutes post-injection for the 1.0 mg/kg dose of nicotine were 10 ng/ml higher compared to the 0.6 mg/kg dose at the same time point (Figure 18b). The linear trend of this change in brain nicotine levels suggested that brain nicotine levels following a lower nicotine dose, 0.3 mg/kg, would be quantifiable at 15-30 minutes post-injection (Figure 18b); however, brain nicotine levels were below the limit of detection at all time points measured using 0.3 mg/kg nicotine (data not shown). This nicotine dose is much higher than the dose given IV (0.1 mg/kg), which yielded detectable brain nicotine levels, suggesting that brain nicotine levels are lower following a SC

nicotine injection. This difference could be due to the slower absorption phase with SC injection compared to IV (reducing peak concentrations), where nicotine deposited into the fat layer between skin and muscle must then enter blood circulation before entering the brain (Kalant, 2007). With IV injection, nicotine is injected directly into blood so it can rapidly enter the brain resulting in higher peak concentrations (Kalant, 2007). A repeated measure ANOVA of 0.6 and 1.0 mg/kg nicotine revealed a significant effect of dose on nicotine levels ($F[1,24]=8.3$, $p < 0.01$). Post-hoc analysis revealed significant differences between the two doses at all time points starting from 15 minutes post-injection ($p < 0.05$). These results demonstrate that nicotine brain levels can be quantifiable following a subcutaneous route of administration and can change proportionately with the peripheral dose given.

Brain cotinine levels were quantifiable but low (< 10 ng/ml), and a repeated measures ANOVA did not show a significant effect of dose ($F[1,24]=2.8$, $p = 0.10$). Consistent with that seen after IV administration, the other nicotine metabolites analyzed, including nornicotine, 3-hydroxycotinine, norcotinine, and nicotine-N-oxide, were below the limit of detection (1 ng/ml).

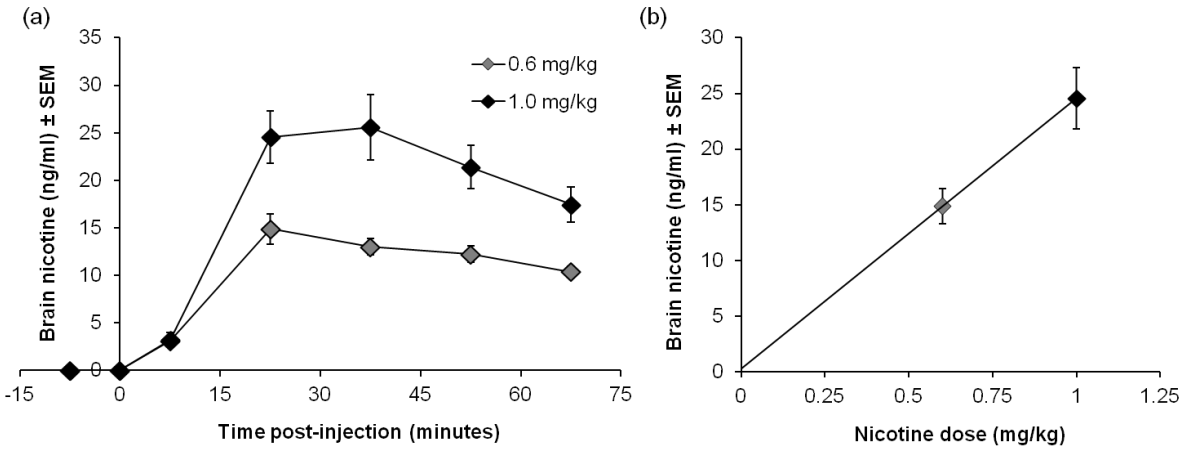

Figure 18. Brain nicotine levels measured by *in vivo* microdialysis increase dose-dependently after a SC injection of nicotine. **(a)** Brain nicotine levels over time after a SC injection of 0.6 or 1.0 mg/kg nicotine. Nicotine levels peaked 30-45 minutes post-injection for the 1.0 mg/kg dose while nicotine levels peaked 15-30 minutes post-injection for the 0.6 mg/kg dose. **(b)** Brain nicotine levels between 0.6 mg/kg and 1.0 mg/kg nicotine doses at the same time point (15-30 minutes post-injection) show a linear trend, demonstrating that brain nicotine levels measured by

microdialysis following SC administration are consistent with increasing peripheral dose. SEM = standard error of the mean

3.2.2 Nicotine brain levels following 7 days of nicotine treatment were lower compared to brain levels measured prior to treatment

Nicotine brain levels in the right lateral ventricle, measured after 7-day nicotine treatment (labeled “Chronic”), displayed a different pattern over time compared to nicotine brain levels measured without prior exposure to nicotine (labeled “Acute”) (Figure 19a). A repeated measures ANOVA analysis revealed a significant interaction between nicotine exposure (Acute vs. Chronic) and time ($F[2.8, 39.1]= 4.1$, $p = 0.01$). The nicotine brain levels collected from 15-45 minutes after the nicotine challenge injection were lower in the chronic condition compared to the acute condition, where a paired t-test comparing total nicotine levels within this time frame showed that this difference was significant ($t[19]= 2.1$, $p = 0.04$) (Figure 19b). At subsequent time points nicotine brain levels were not different between acute and chronic nicotine conditions (Figure 19a). Plasma levels of nicotine collected at 22.5 and 112.5 minutes post-injection were not different between acute and chronic conditions (Figure 19c), confirming that peripheral nicotine levels were not altered by the 7-day nicotine treatment. This suggests that 7-day nicotine treatment increased CYP2B activity in the brain, resulting in greater metabolism of nicotine and a reduction in nicotine levels.

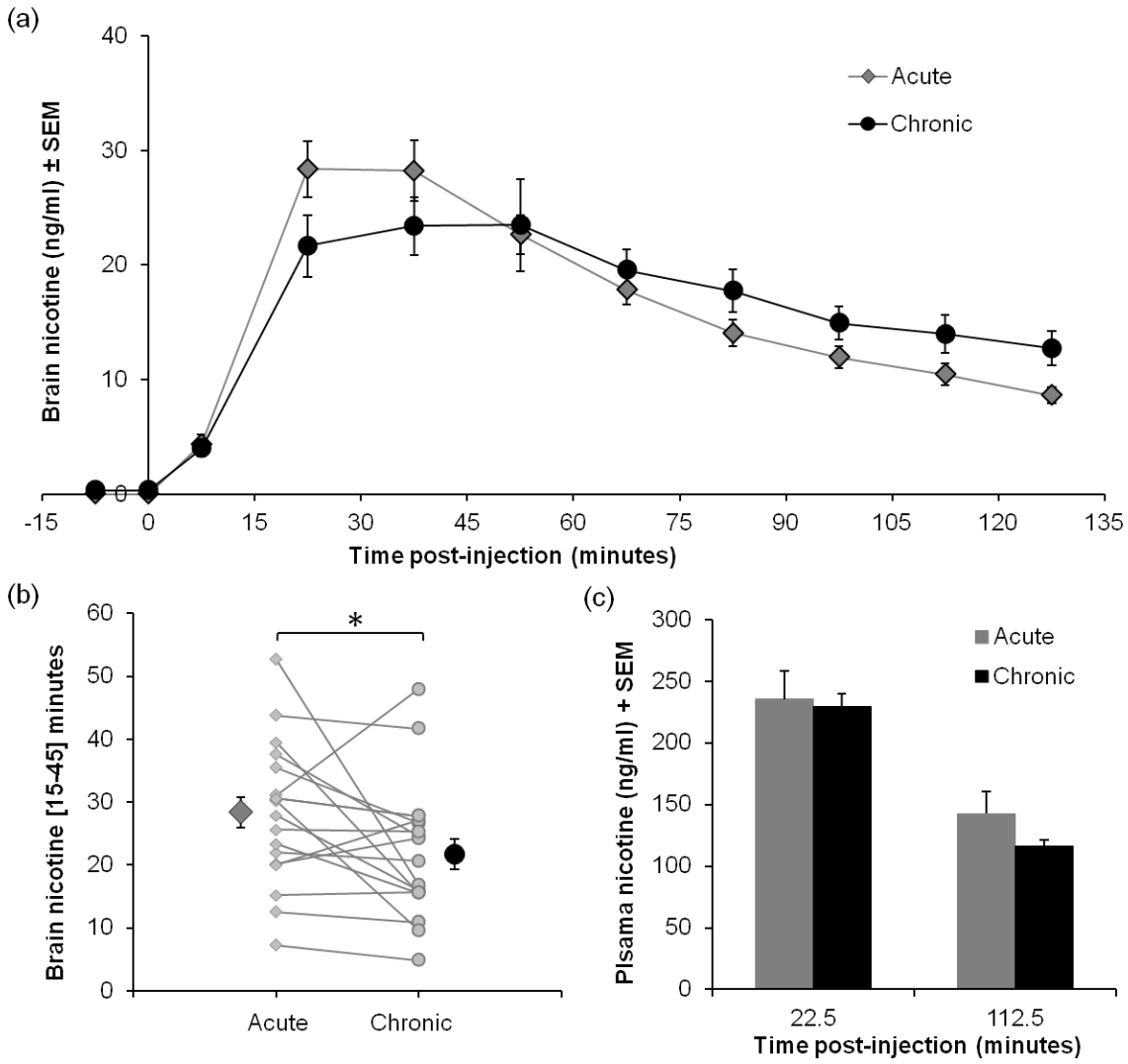

Figure 19. Nicotine brain levels were reduced after chronic nicotine treatment. **(a)** Mean nicotine brain levels over time post-injection measured prior to 7-day nicotine treatment (acute condition) and after 7-day nicotine treatment (chronic condition). **(b)** Nicotine brain levels collected within 15-45 minutes post-injection in acute vs. chronic conditions. Individual animal data is represented by the gray circles and mean data for acute vs. chronic conditions are represented by the gray diamond and black square respectively. **(c)** Mean plasma nicotine levels collected at 22.5 and 112.5 minutes post-nicotine injection were not significantly different between acute and chronic conditions. SEM = standard error of the mean. *$p < 0.05$

3.2.3 Nicotine brain levels after a 2 week period with no nicotine exposure were higher compared to brain levels after post-nicotine 7-day treatment

Following the 7-day nicotine treatment, animals had two weeks in their home cages prior to a third nicotine microdialysis collection under the same protocol used in the acute and chronic conditions. A number of animals had lost their microdialysis cannula or were lost to illness; this resulted in eight animals with patent cannulas. For these eight animals which are the subset tested for washout, the acute and chronic conditions are shown for within animal comparison to the washout condition to illustrate the effect of 7-day nicotine exposure and 14-day washout. Nicotine brain levels, measured in the right lateral ventricle, between acute vs. chronic conditions in the eight animals (Figure 20a) showed nicotine levels over time consistent with that seen with all animals (n = 20) (Figure 19a). Nicotine brain levels collected 15-45 minutes post-injection were significantly lower in the chronic condition compared to acute in all animals (Figure 19b); therefore using paired one tail t-tests with the data from the eight animals, nicotine levels collected within this time frame were also significantly lower in the chronic condition compared to acute ($t[7]= 2.29$, $p = 0.03$) (Figure 20b). Thus, the observed reduction of brain nicotine levels following 7-day nicotine treatment also occurs within this subset of animals and data from the chronic condition can be compared to the 2-week washout to determine whether brain nicotine levels remain similar to that in the chronic condition. If there is no change, it would indicate that brain CYP2B is still induced.

To examine whether the 7-day nicotine treatment, which can induce brain CYP2B and lower nicotine brain levels, remained following 2 weeks without nicotine treatment, nicotine brain levels in the right lateral ventricle were measured again 2 weeks later. Nicotine brain levels over time, following the same 1 mg/kg/SC nicotine challenge dose, were different after the two week washout period compared to the chronic condition (Figure 21a). Repeated measures ANOVA revealed a significant effect of nicotine treatment (Chronic vs. Washout, $F[1,7]=11.44$, $p = 0.01$), showing that nicotine brain levels were higher after the washout period compared to the chronic condition. There was also a significant interaction between nicotine treatment and time ($F[8,56]=6.15$, $p < 0.01$), showing that nicotine levels over time were significantly different after the washout condition compared to the chronic condition. Nicotine brain levels over time in the acute condition compared to washout were not significantly different (data not shown together). The same time frame post-nicotine injection examined for acute vs. chronic conditions was also

compared for chronic vs. washout conditions. Brain nicotine levels collected within 15-45 minutes post-injection were significantly higher in the washout condition compared to chronic (Figure 21b, $t[7]=2.71$, $p = 0.02$), showing that the reduction in nicotine brain levels following 7-day nicotine treatment can return to pretreatment levels following at least 2 weeks without nicotine treatment, suggesting that brain CYP2B levels had returned to pre-7-day nicotine treatment levels. Plasma nicotine levels in the washout condition were similar to acute and chronic conditions, demonstrating that the differences in nicotine levels over these different conditions were only observed in the brain (Figure 21c). These results indicate that increased nicotine metabolism by brain CYP2B induction does not persist past 2 weeks, which is consistent with the observation that CYP2B protein levels return to baseline after 7 days from the end of nicotine treatment (Khokhar, 2010).

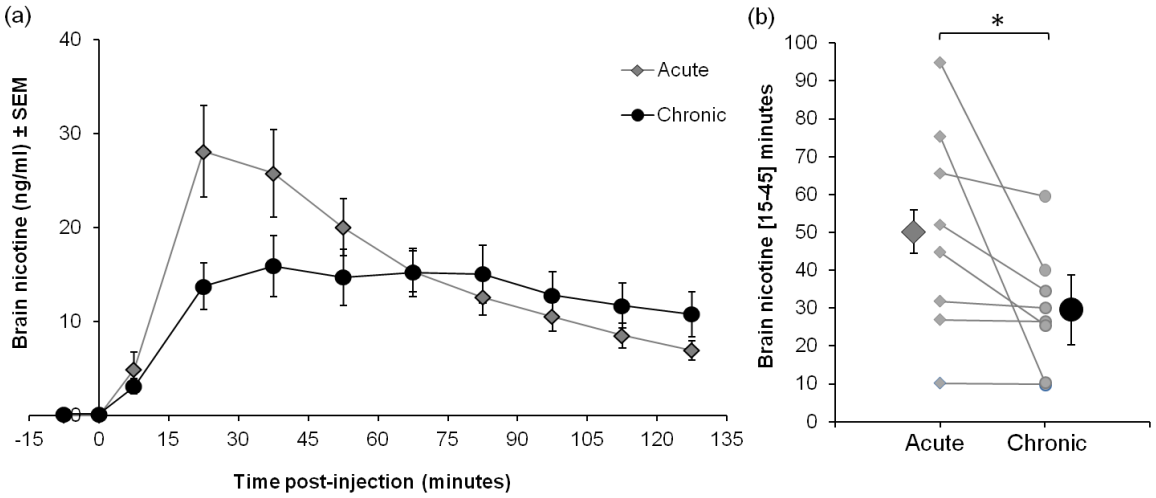

Figure 20. Brain nicotine levels before (acute) and after (chronic) 7-day nicotine treatment in animals that underwent the 2-week washout. **(a)** Mean brain nicotine levels measured over time post-injection for the eight animals display the same reduction previously reported in Figure 17a. **(b)** Within-animal, the reduction in brain nicotine levels collected within 15-45 minutes post-injection was also observed in these eight animals. SEM = standard error of the mean. * $p < 0.05$

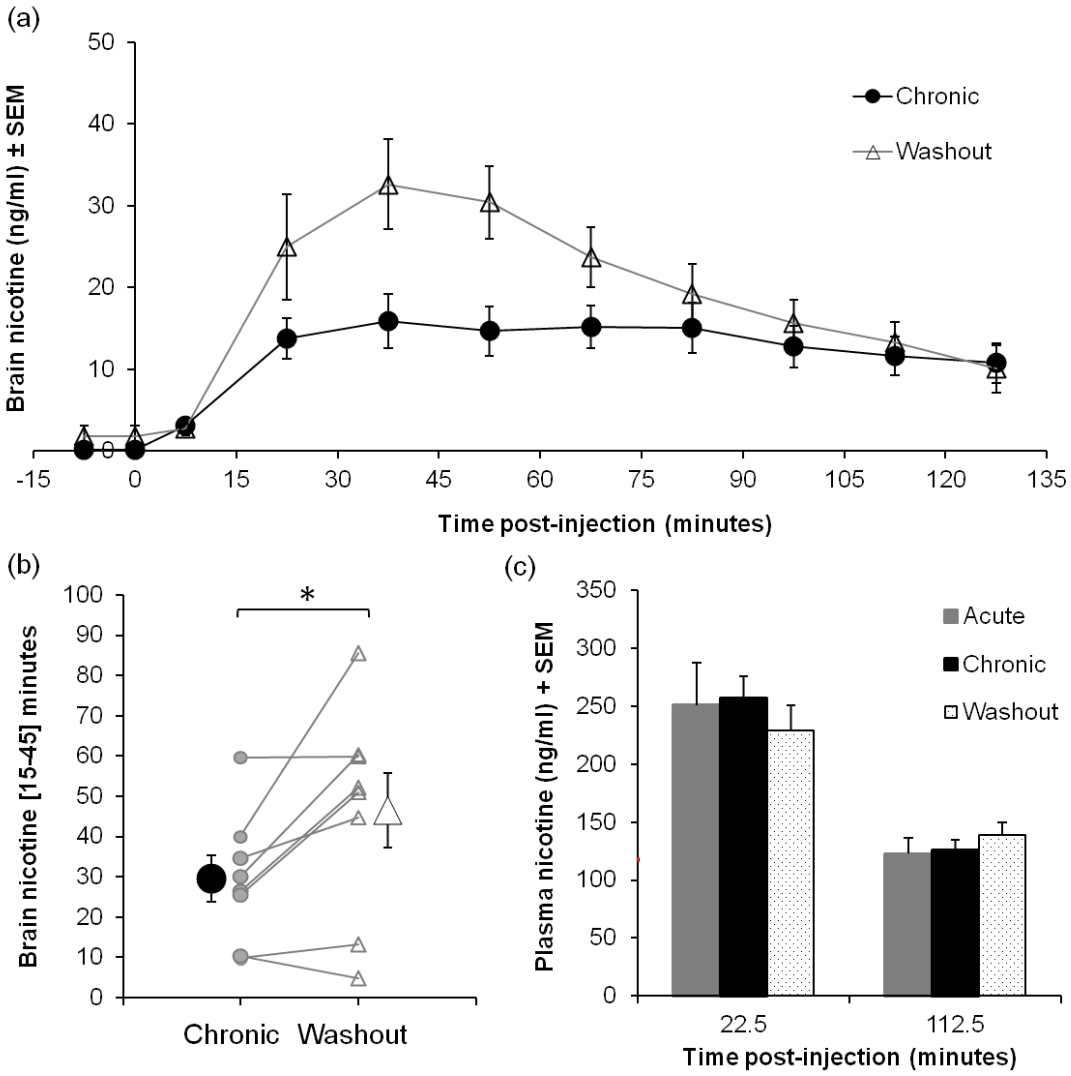

Figure 21. Brain nicotine levels before (chronic) and after (washout) 2-week washout period. **(a)** Mean brain nicotine levels measured over time post-injection for the eight animals display different patterns in the chronic and washout conditions. **(b)** Within-animal, the reduction in brain nicotine levels collected within 15-45 minutes post-injection in the chronic condition was not observed in the washout condition, where nicotine levels were significantly higher than that seen in the chronic condition. **(c)** Mean plasma nicotine levels at 22.5 and 112.5 minutes post-injection after acute, chronic, and washout conditions. There was no difference in plasma levels between any of the 3 conditions at both time points. SEM = standard error of the mean. *$p < 0.05$

3.2.4 ICV injection of C8X increased nicotine brain levels after a single SC injection of nicotine

To show that the decreases in nicotine brain levels following a 7-day nicotine treatment were due to CYP2B induction, the effect of the CYP2B inhibitor was examined before and after 7-day nicotine treatment. In Section 3.1, a single C8X ICV injection significantly increased nicotine brain levels compared to ACSF ICV injection following an IV injection of nicotine. Therefore, to test whether a single C8X ICV injection could increase nicotine brain levels following a SC injection (1 mg/kg challenge dose), animals were given the same dose of either C8X (20 µg) or ACSF 22 hours prior to nicotine microdialysis. Figure 22a shows nicotine levels from the right lateral ventricle, collected over time, for both C8X and ACSF groups. Nicotine brain levels were higher in C8X-treatment compared to ACSF-treatment (Repeated measures ANOVA, $F[1,15]=7.28$, $p = 0.02$). There was also a significant interaction between ICV treatment and time ($F[2.8,42.15]=5.34$, $p < 0.01$), indicating that nicotine levels over time were different with C8X treatment compared to ACSF. Total nicotine collected from the brain 15-135 minutes post-injection was significantly higher with C8X ICV treatment compared to vehicle (Figure 22b, $t[15]= -2.97$, $p < 0.01$). These results are consistent with increased brain nicotine levels with C8X ICV pre-treatment following an IV injection (Section 3.1.2) and provide additional evidence that inhibiting CYP2B activity by C8X can increase nicotine levels in the brain. ICV treatment did not influence plasma nicotine (Figure 22c), suggesting that higher nicotine levels found in the brain were due to C8X inhibiting CYP2B activity in the brain and not in the liver.

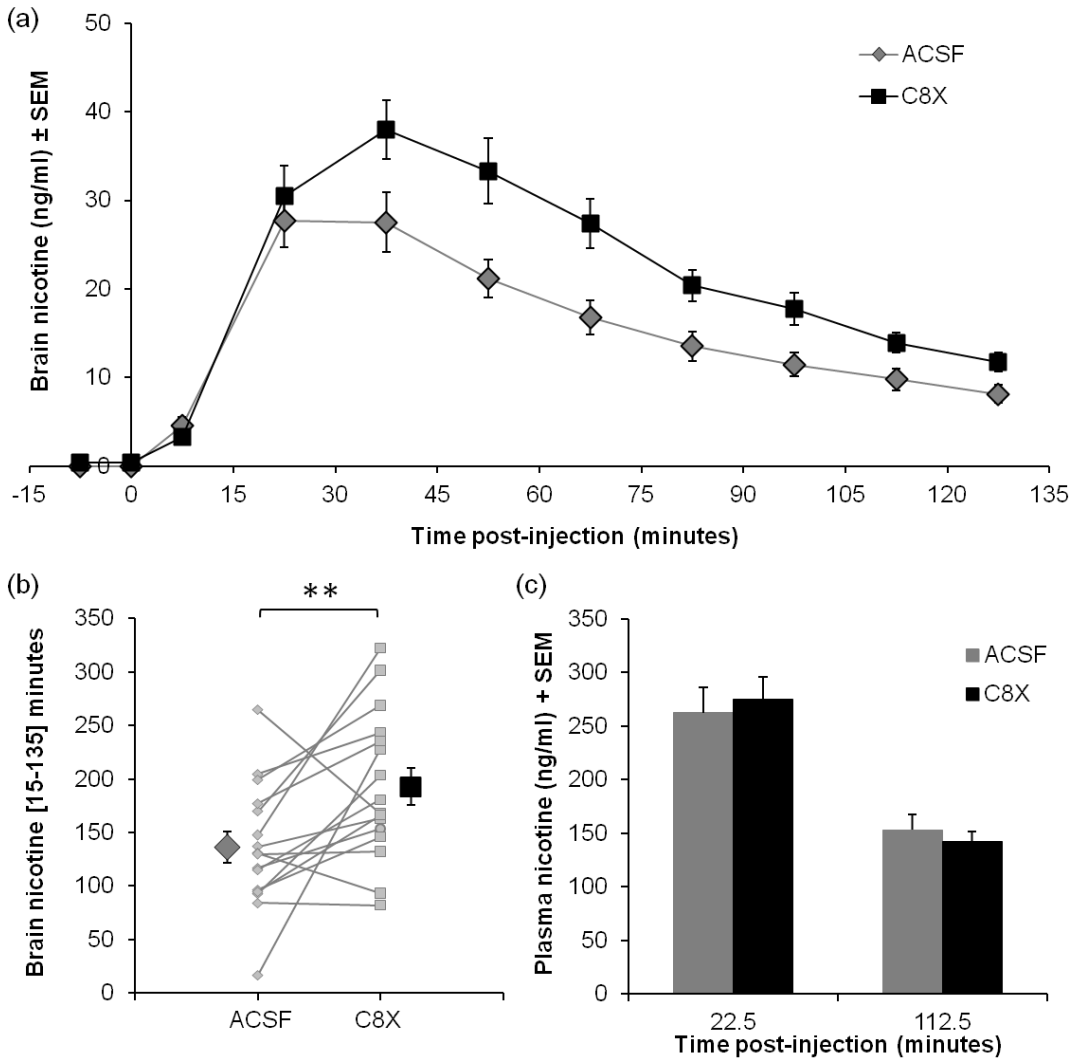

Figure 22. Brain nicotine levels after a single ICV injection of C8X or ACSF in rats without prior exposure to nicotine (acute condition). **(a)** Mean brain nicotine levels over time post-injection were higher with an C8X compared to ACSF (n = 16/ICV group) **(b)** Within-animal, total nicotine collected from 15-135 minutes post-injection was higher with C8X ICV injection compared to ACSF ICV injection **(c)** Mean plasma nicotine levels collected at the two blood time points were not different between ICV treatments at either time point. SEM = standard error of the mean.**$p < 0.01$

To determine whether injecting C8X ICV during the 7-day nicotine treatment would inhibit the potentially induced CYP2B, animals were given C8X or ACSF ICV injections of the same dose

(20 µg) daily for the seven days of nicotine injection. Following the 7-day nicotine treatment, mean nicotine brain levels after C8X ICV treatment were not significantly higher than after ACSF ICV treatment, although mean values with C8X compared to ACSF treatment were in the expected direction of higher nicotine levels with C8X treatment (Figure 23a). This suggests that while nicotine treatment may have induced CYP2B, the C8X inactivated this induced level of enzyme, which resulted in the change in nicotine brain levels between groups treated with C8X and ACSF. Together with the knowledge from previous studies that a 7-day nicotine treatment induced a functional CYP2B in brain (Khokhar, 2010), the data shown in Figure 23a suggest that ICV inhibitor administration blocked the functional impact of the induced CYP2B. This provides further support that the change in brain nicotine levels following 7-day nicotine treatment was in fact due to induction of CYP2B. Mean plasma nicotine levels were not different with C8X and ACSF ICV treatments (Figure 23b), indicating that ICV treatments did not alter peripheral nicotine metabolism and suggesting that increased brain nicotine levels following inhibitor treatment were due to brain CYP2B inhibition.

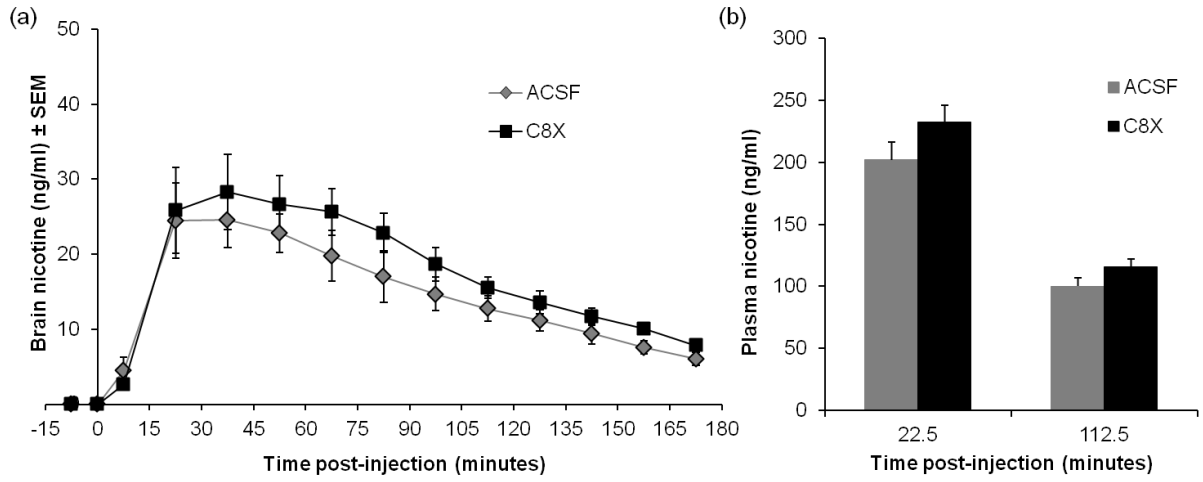

Figure 23. Brain nicotine levels after repeated ICV injection of C8X or ACSF in rats after 7-day nicotine treatment (chronic condition). **(a)** Mean brain nicotine levels over time post-injection were not significantly higher in rats given C8X ICV injections compared to rats given ACSF ICV injections. **(b)** Mean plasma nicotine levels collected at the two blood time points were not different between ICV treatments. SEM = standard error of the mean

In a pilot experiment, another small set of animals (n = 3) underwent 7-day nicotine treatment with nicotine microdialysis carried out on day 1 (acute) and day 7 (chronic) of nicotine treatment however on day 6, rats received a single ACSF ICV injection 22 hours prior to their nicotine microdialysis on day 7. The day after chronic nicotine microdialysis (day 8) animals were given an additional nicotine injection (1 mg/kg/SC) to maintain consistent exposure to nicotine for CYP2B induction, and then received a single C8X ICV injection. The next day (day 9), approximately 22 hours after their C8X ICV injection, rats underwent another nicotine microdialysis session with the 1 mg/kg/SC nicotine challenge injection. In this within-animal study, the 7-day nicotine treatment reduced brain nicotine levels (acute vs. chronic) consistent with that seen in the previous cohorts (Figure 24a). Brain nicotine levels (day 9) following C8X pretreatment were higher than the chronic condition, which suggests that C8X inhibited the induced CYP2B activity, thus resulting in higher nicotine levels (Figure 24b). Brain nicotine levels were lower than that in the acute condition, which suggests that the dose of C8X did not completely inhibit the CYP2B that would be increased by 7-day nicotine treatment (Figure 24c). These preliminary findings with CYP2B inhibition after 7-day nicotine treatment indicate that the reduction in brain nicotine levels is mediated, at least in part, by elevated CYP2B activity in the brain. Therefore, the three different experiments with C8X ICV treatment, where C8X was given acutely, throughout 7-day nicotine treatment, and acutely after 7-day nicotine treatment, were able to show changes in nicotine levels in the direction expected if CYP2B was inhibited. This suggests that altered CYP2B activity in the brain can influence nicotine levels following systemic administration of nicotine.

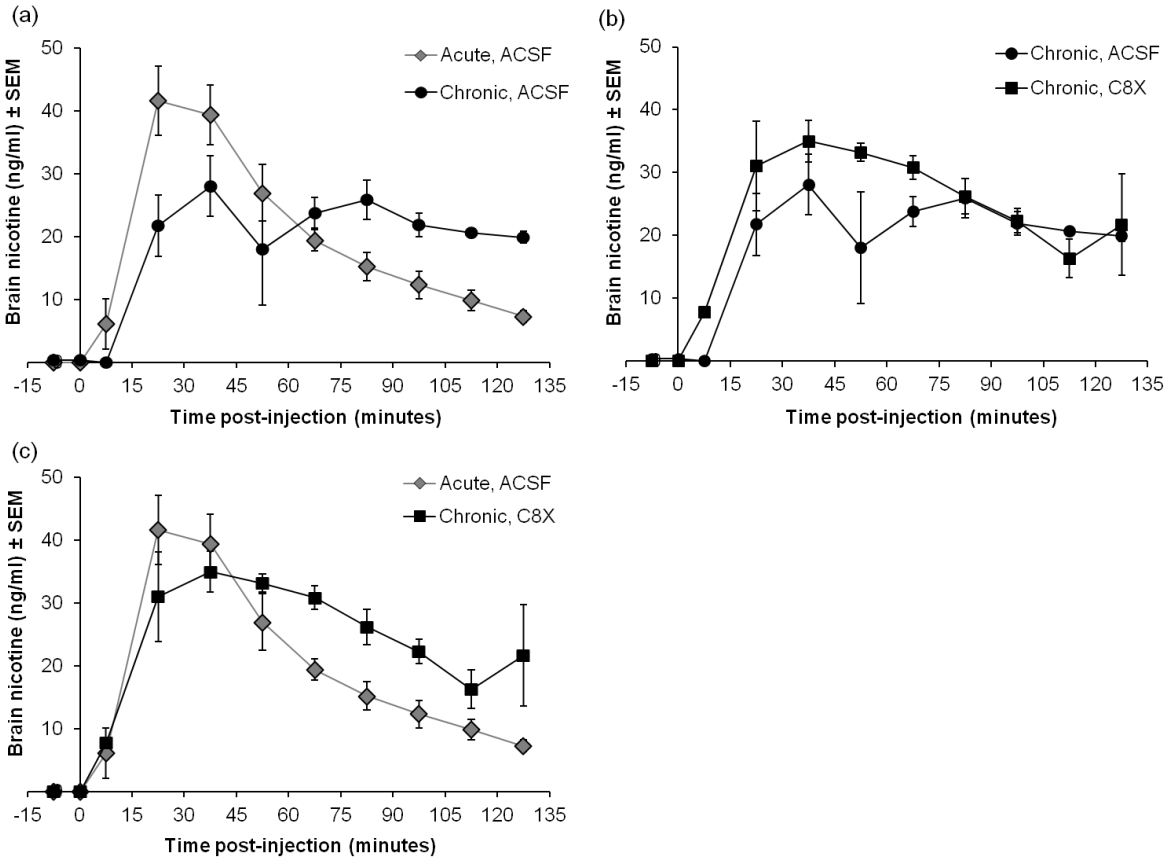

Figure 24. Brain nicotine levels after a single C8X ICV injection in rats given a 7-day nicotine treatment (chronic condition). **(a)** Rats that were treated with 7 days of nicotine displayed the reduction in brain nicotine levels (chronic) compared to before chronic nicotine exposure (acute) within the 15-45 minutes post-injection. **(b)** After C8X ICV injection, brain nicotine levels increased compared to nicotine levels in chronic condition. This elevation in brain nicotine levels can be observed from 15-75 minutes post-injection. **(c)** Brain nicotine levels following C8X ICV injection were lower than that in the acute condition within the 15-45 minutes post-injection, where nicotine levels in the chronic condition were lower compared to acute. SEM = standard error of the mean

Section 4: Discussion

1 Effect of food training on nicotine self-administration

1.1 Acquisition of NSA

The investigation of food training and nicotine dose on NSA behaviour yielded observations for acquisition that have not been reported in the NSA literature. Dose-response information for acquisition of NSA behaviour was generated for both animals that underwent prior food training and animals that did not undergo food training (spontaneous acquisition). Prior food training facilitated acquisition at nicotine doses (3.75 and 7.5 µg/kg) where only half of rats acquired NSA in the spontaneous acquisition group. In contrast, rats that underwent spontaneous acquisition displayed dose-dependent increases in acquisition. This demonstrates that food training is one factor that can influence acquisition behaviour in NSA, particularly at low nicotine infusion doses that may not reliably establish NSA, and that spontaneous acquisition reveals the dose-dependent effect of nicotine to establish self-administration behaviour.

Food training is commonly used to facilitate acquisition of NSA and was used in the first publications that characterized the limited access paradigm for NSA (Corrigall and Coen, 1989; Donny et al., 1995). Therefore, the enhancement of acquisition with food training in these experiments is consistent with that seen in the literature. What is novel is the comparison of food training to spontaneous acquisition at the same nicotine doses; only one study to date has directly compared food training vs. no food training (Clemens et al., 2010). In this study acquisition was not tested because animals were trained with 30 µg/kg, which is the nicotine dose commonly used to establish NSA (Clemens et al., 2010). A few studies have reported NSA with spontaneous acquisition in limited access schedules (Shoaib et al., 1997; Shram et al., 2008b; Peartree et al., 2012), however 15 µg/kg was the lowest dose used. These studies found dose-dependent increases in acquisition from 15 to 30 or 60 µg/kg. In the current study 3.75 and 7.5 µg/kg doses of nicotine were used, which extends the dose-response relationship for NSA acquisition at lower doses. Thus, the acquisition data from 3.75-15 µg/kg establishes a dose range where acquisition is ascending and is dependent on infusion dose. This dose-response relationship in acquisition for nicotine is consistent with that seen in other drugs of abuse. Dose-

dependent increases in spontaneous acquisition of the psychostimulants cocaine and amphetamine have also been reported (van Ree et al., 1978; Carroll and Lac, 1997) and food training can also enhance acquisition of these drugs (Weiss et al., 2000; Di Ciano et al., 2001; Mantsch et al., 2001; Green et al., 2002; Bongiovanni and See, 2008). The doses of 15 µg/kg and above (30 µg/kg) resulted in the majority of animals acquiring NSA, which indicates that these infusion doses are reinforcing enough to establish NSA and food training is not needed to facilitate this behaviour.

There were other differences in NSA between food trained and spontaneous acquisition animals, such as session length (1 hour vs. 2 hours) and access (5 days a week vs. 7 days a week daily sessions). Therefore enhanced acquisition with food training could also be due to the food trained animals having less access to SA. When another cohort of animals were tested head-to-head with the 7.5 µg/kg dose, the greater acquisition observed with food training compared to spontaneous acquisition at this dose was replicated, demonstrating that enhanced acquisition at this dose by food training was not influenced by session length and access. Thus, the effect of food training on acquisition can be attributed to food training independent of the other differences in NSA parameters.

Secondary reinforcers (cues) can also influence NSA behaviour (Caggiula et al., 2001; Caggiula et al., 2002). In addition to nicotine's primary reinforcing effect it also enhances the reinforcing effect of stimuli presented when it is administered (Caggiula et al., 2009). Virtually no animals that were food trained or underwent spontaneous acquisition for cue self-administration without nicotine acquired the behaviour, which demonstrated that these animals were not responding for the cues independent of nicotine. This suggests that facilitation of acquisition by food training, particularly at the low nicotine infusion doses where facilitation occurred, was not due to food training enhancing the reinforcing effect of the cues. It also indicates that the animals in the spontaneous acquisition groups that acquired NSA at low nicotine infusion doses were responding for nicotine as opposed to responding for the cues. Peartree et al. (2012) was able to establish NSA behaviour without prior food training or the presence of cues using doses from 15-60 µg/kg, demonstrating that nicotine can establish self-administration without the addition of reinforcement enhancing conditions.

Once NSA was established, total mean reinforcements were not different between food trained and spontaneous acquisition rats that acquired the behaviour. This indicates that responding was driven by the infusion dose of nicotine after NSA had been established. Clemens et al. (2010) also found similar mean reinforcements between animals food trained and not food trained after acquisition, even when a dose-response curve using 7.5, 15, 30, or 60 µg/kg doses was generated in these two groups. Therefore, while food training can enhance acquisition of NSA it does not appear to influence responding for nicotine once the behaviour has been established.

One limitation of our experiment is that the acquisition results are based on an arbitrary set of acquisition criteria. These criteria are based on a previous study that used the same rat strain and NSA parameters (Shram et al., 2008b); however, we acknowledge that our findings are restricted to these conditions. This is one general limitation in the NSA literature as there is no standard set of criteria for acquisition. Animals that did not meet acquisition criteria in our experiments displayed low and/or decreased responding as NSA sessions progressed and did not show preference for the active lever, which are two conditions characteristic of NSA behaviour. Therefore, these differences in NSA behaviour between animals that did not acquire vs. animals that did provide confidence that the criteria used reasonably identified NSA acquisition.

1.2 Nicotine-seeking behaviour

When animals responded under the PR schedule, animals that food trained reached higher final ratios of responding for one nicotine infusion compared to animals that underwent spontaneous acquisition. The PR schedule is considered a measure of the reinforcing efficacy of the drug because it quantifies the amount of work the animal is willing to do in order to obtain the drug (Richardson and Roberts, 1996). Therefore, if food trained animals responded more for nicotine then it suggests that food training increased the motivation to continue with the behaviour.

These findings are interesting given that food training did not influence maintenance behaviour under an FR schedule after animals acquired NSA. It suggests that prior food training provides additional motivation to that from nicotine infusion, possibly by making the cues more salient or creating an association of food with the operant chamber. Animals that are food trained with cues present increase their responding for nicotine as well as saline (Clemens et al., 2009; Clemens et al., 2010), suggesting that food training may contribute to cue reinforcement. In our food training protocol animals were not exposed to any cues, which indicate that it is unlikely that food

training made cues more salient. Furthermore, in the cue without nicotine SA experiments, there was no difference in responding under the PR schedule between rats that food trained or that underwent spontaneous acquisition. Final ratios completed in the cue without nicotine condition were significantly lower than those reported with the lowest nicotine infusion dose (7.5 µg/kg), indicating that the cues without nicotine were not as reinforcing as cues with nicotine. Therefore, responding for nicotine under the PR schedule in these experiments was due to nicotine's reinforcing effect and the increase in motivation with food training compared to spontaneous acquisition in the analysis of separate animal cohorts may be independent of food reinforcement.

One of the limitations of this analysis was that other study design factors were also different between food trained and spontaneously acquired rats, which were mentioned in the previous section. It is possible that these differences (shorter session length and 5 continuous sessions with 2 days off with food training) may have lead to altered motivation. The data from the head-to-head experiment where rats self-administered nicotine under identical conditions is consistent with this explanation. When all factors except prior food training were the same, final ratios completed under the PR schedule were not different between food trained and spontaneously acquired rats. Food trained animals had 1 hour sessions, therefore it is possible that because they learned to respond within a shorter period of time compared to spontaneous acquisition animals (2 hours), they were more motivated to respond. This is consistent with the similar levels of total nicotine infusions earned during FR; animals that underwent spontaneous acquisition had twice the time to obtain nicotine and thus earned half of their total reinforcements within the first hour. Therefore, food training alone does not increase the motivation to respond for nicotine.

It is interesting to note that in the head-to-head experiments the opposite is observed between food training and spontaneous acquisition under the PR schedule, where final ratios completed were higher in animals that underwent spontaneous acquisition. Although it was not statistically significantly different, it suggests that motivation might be lower with prior food training, possibly due to the extinction of food association with the active lever. Another possibility is that animals that underwent spontaneous acquisition were more motivated to respond for nicotine because they were able to acquire without prior training. This suggests that underlying biological differences could exist in rats that readily acquire at the lower nicotine infusion doses compared to rats that require food training. Animal models are used under the assumption that the animals are biologically identical; however, behavioural differences within inbred rat strains have been

characterized, such as differences in locomotor activity and response to novel environments (Dellu et al., 1996), and these differences can influence the response to drugs of abuse such as amphetamine (Dellu et al., 1996) and cocaine (Hooks et al., 1991).

The effect of food training on PR responding for nicotine or other drugs of abuse has not been reported in the literature. Other factors such as drug pre-treatment can influence responding under the PR schedule for other drugs of abuse: amphetamine pre-treatment can increase breakpoints (the final ratio completed) in amphetamine self-administration (Mendrek et al., 1998; Lorrain et al., 2000). The Clemens et al. study (2010), which directly compared NSA after food training vs. spontaneous acquisition, did not test PR breakpoint; however, nicotine-seeking behaviour measured by extinction and nicotine reinstatement was tested. Food training did not affect extinction behaviour but did result in higher responding when the cue was presented or when animals received a priming injection of nicotine (0.3 mg/kg/SC) (Clemens et al., 2010). In our head-to-head experiment, there was no effect of food training on extinction, consistent with the Clemens study (2010); however, there was also no effect of food training on cue or nicotine reinstatement. The training dose for this current study (7.5 µg/kg) was lower than that in Clemens (2010) (30 µg/kg), therefore it is possible that the effect of food training at the lower nicotine dose was not as reinforcing as that with the higher dose in the previous study. The effect of food training on cocaine-seeking behaviour was examined in Bongiovanni and See (2008) where no differences were found in extinction or reinstatement with cues or cocaine, which is consistent with our results. These results suggest that the effect of food training on cue reinstatement may be training dose-dependent, where food training may have no effect on lower infusion doses such as that used in the current study but may have an enhancing effect on higher infusion doses such as that used in Clemens et al. (2010). Thus, for low training doses where food training facilitates acquisition, food training might not influence measures of nicotine-seeking behaviour.

1.3 Relevance to acquisition of human smoking

Food training is used to enhance learning the response that elicits reward. Thus in NSA, once this association is established the reward is changed from food to a nicotine infusion and if the infusion dose is rewarding the behaviour continues (Clemens et al., 2010). Responding decreases during NSA acquisition under the FR1 schedule in FT rats, where active lever responding was

highest in the first two sessions but lower during stable responding in subsequent sessions, suggesting that rats adjusted from responding for food to responding for nicotine (Garcia et al., 2014). Learning to respond for nicotine by non-nicotine factors is also observed in human smokers. Non-drug factors can also influence smoking initiation, such as smoking by peers or family members and by stress (Mayhew et al., 2000; Wang et al., 2000), suggesting that people try cigarettes for reasons independent of nicotine reward. After smoking initiation, the reinforcing effect of nicotine is believed to maintain the behaviour, which might subsequently lead to nicotine dependence (Rose and Corrigall, 1997; Wang et al., 2000). Therefore, similar to responding for food reward transitioning to responding for nicotine during NSA acquisition in rats, human smokers may start smoking because of social pressure or stress but continue smoking for the reinforcing effect of nicotine.

Our spontaneous acquisition experiments also demonstrate that NSA can be established at nicotine infusion doses lower than doses previously reported where spontaneous acquisition was used (Shoaib et al., 1997; Shram et al., 2008b; Peartree et al., 2012), extending the dose-response curve to reveal an ascending dose-dependent acquisition. These findings provide nicotine doses where some, but not all, rats acquire NSA, which is useful for investigating factors that influence acquisition or known populations that are more vulnerable to becoming smokers. Impulsivity is a predictor of smoking and alcohol use (Granö et al., 2004), suggesting that people who score high in impulsivity are at a greater risk for developing drug dependence; this trait can be modeled in rats and can increase NSA behaviour (Diergaarde et al., 2008b). Thus, biological factors that increase the vulnerability to nicotine dependence could be ascertained using NSA at low nicotine doses.

Low infusion doses of nicotine may be closer to the amount of nicotine smokers receive from cigarettes. One cigarette can deliver between 10-30 µg/kg of nicotine, whereby people take on a range of 10-20 puffs per cigarette resulting in 1-3 µg/kg of nicotine delivered by each puff (Matta et al., 2007; Sorge and Clarke, 2009). Thus, the infusion dose commonly used to establish NSA in rats, 30 µg/kg, is similar to rats receiving one cigarette. In our spontaneous acquisition experiments, 3.75 and 7.5 µg/kg nicotine doses were able to establish NSA in a number of rats; these doses are closer to the amount of nicotine delivered in one or two cigarette puffs. These findings suggest that spontaneous acquisition of NSA with lower infusion doses might be a better model to investigate human smoking acquisition and initiation behaviour.

2 Effect of brain CYP2B inhibition on nicotine self-administration

2.1 Acquisition

The observed dose-dependent acquisition of NSA with spontaneous acquisition in Section 1 established a range of nicotine infusion doses by which a proportion of animals acquired the behaviour. These doses were subsequently used to determine whether inhibiting brain CYP2B with C8X could alter acquisition. C8X ICV treatment increased the proportion of rats that acquired NSA compared to vehicle ICV (ACSF) treatment. This effect was significant in animals trained with 7.5 µg/kg nicotine, the dose where approximately half of animals acquired NSA with spontaneous acquisition. This dose resulted in the majority of rats acquiring NSA with inhibitor ICV treatment whereas the 15 µg/kg nicotine dose resulted in the most rats acquiring with vehicle ICV treatment, suggesting that the inhibitor produced a leftward shift in the acquisition dose-response curve. Leftward shifts are considered a sensitization of dose, whereby a given response is elicited at a lower dose, which is thought to identify populations that may be more vulnerable to the reinforcing effect of the drug (Deminiere et al., 1989). More animals acquired NSA with C8X ICV treatment at a given nicotine dose, suggesting that the inhibitor increase nicotine's reinforcing effect. C8X, through inhibiting brain CYP2B activity, would decrease nicotine metabolism in the brain and increase brain nicotine levels, leading to more nicotine available to activate the brain reward system. Therefore, greater nicotine reinforcement might have made these animals more inclined to learning NSA behaviour.

The doses used for NSA result in ascending proportions of animals that acquire (Figure 5). Therefore, the increase in acquisition with C8X ICV treatment with 7.5 µg/kg nicotine is consistent with increases in apparent nicotine dose. There was no effect of C8X ICV treatment on acquisition with the 3.75 and 15 µg/kg doses; however this lack of effect can be explained for both nicotine doses. In vehicle ICV-treated rats, the proportion of rats that acquired NSA with the 7.5 µg/kg dose was not higher than with the 3.75 µg/kg dose, indicating that an increase in the apparent nicotine dose by C8X ICV treatment may not be sufficient to increase acquisition. For the 15 µg/kg dose, the majority of vehicle ICV-treated rats acquired NSA, suggesting that this infusion dose of nicotine is already sufficient to reinforce NSA behaviour enough to maximally establish NSA. The increase in acquisition between 7.5 and 15 µg/kg doses in vehicle

ICV treated groups is significant, thus greater acquisition with inhibitor treatment at the 7.5 µg/kg dose is consistent with animals receiving a higher apparent dose of nicotine.

2.2 Maintenance of NSA behaviour

Once animals acquired NSA there was no effect of C8X ICV treatment on NSA behaviour. This suggests that once the association between active lever and nicotine infusion had been established, persistence of responding and levels of nicotine intake were driven by the nicotine infusion dose. The dose-response curve for nicotine is relatively flat for FR2 in our NSA paradigm (Figure 11) which is consistent with a previously reported dose-response curve under FR5 (Rose and Corrigall, 1997). Mean active lever presses under FR2 were not significantly different between 7.5 and 15 µg/kg infusion doses which indicate that there is little change in responding with a higher infusion dose. Thus, increases in the apparent dose of nicotine with C8X may not result in significant changes in responding during the FR schedule, at least at the doses tested.

The FR schedule can provide information on patterns of drug intake and the rate of responding for the drug; however, it is not universally agreed that FR is a suitable model to investigate a drug's reinforcing efficacy or strength (Arnold and Roberts, 1997). For drugs of abuse the rate of responding has been typically associated with the reinforcing effect of the drug dose, where the rate decreases with higher doses, suggesting that lower rates of responding correspond to greater reinforcement. However, lower rates of responding have also been attributed to a decrease in reinforcement in cocaine self-administration after injection of the neurotoxin 6-hydroxydopamine (6-OHDA) into the nucleus accumbens, which depletes dopamine (Roberts et al., 1980; Arnold and Roberts, 1997). Thus, other schedules within the NSA model, such as the PR schedule, are more reliable to investigate the strength of the drug's reinforcement (Richardson and Roberts, 1996; Arnold and Roberts, 1997). Drugs affecting specific stages of NSA, but not maintenance behaviour in self-administration, have been reported in the literature. Venlafaxine, a dual serotonin and norepinephrine re-uptake inhibitor, can reduce acquisition of heroin self-administration with no effect on maintenance under an FR schedule (Magalas et al., 2005). VDM11, an anandamide re-uptake inhibitor, can reduce nicotine reinstatement behaviour with no effect on maintenance behaviour (Gamaleddin et al., 2011). Nicotine can act on serotonin and norepinephrine systems in addition to dopamine (Picciotto and Corrigall, 2002;

Danielson et al., 2011), whereby acute nicotine can elicit serotonin and norepinephrine release in limbic and frontal cortex regions (Picciotto and Corrigall, 2002). Conversely, acute and chronic nicotine treatment can also modulate monoamine transporter activity leading to increased uptake of monoamines (Danielson et al., 2011), which suggests compensatory mechanisms following nicotine's activational effect on these systems. The endocannabinoid system is considered a component of brain reward and motivational behaviour; several studies have shown that altering this system can influence nicotine-reinforced behaviour (Scherma et al., 2008) in addition to the reinforced behaviour of other drugs of abuse (Panagis et al., 2014). Thus, the different effects of altering these central neurotransmitter systems on select stages of NSA, along with our results, suggest that the impact of nicotine infusions and mechanisms underlying nicotine reinforcement may be different within each stage of NSA.

2.3 Nicotine-seeking behaviour

C8X ICV treatment increased breakpoints (final ratios completed) under the PR schedule. Although the effect of C8X treatment was statistically significant at one nicotine infusion dose (15 µg/kg), mean final ratios were higher at all doses with C8X ICV treatment compared to vehicle. This suggests that C8X, by reducing brain CYP2B activity, increased brain nicotine levels following each infusion leading to greater nicotine reinforcement. Because response demand increases after one infusion is earned, the interval of nicotine intake is not stable, and this creates similar conditions to that in acquisition for the effect of C8X on brain nicotine levels. During PR animals averaged between five to fifteen infusions over the 2 hour session, which is lower than the amount of infusions (>20) they earned during FR NSA. Therefore, greater reinforcement from receiving one infusion with C8X ICV treatment would increase the motivation to respond for another infusion, leading to more infusions earned.

When access to nicotine was removed during extinction, animals treated with C8X ICV required more sessions to meet extinction criteria compared to vehicle. It is likely that C8X ICV treatment increased brain nicotine levels by reducing brain nicotine metabolism during NSA. Higher brain nicotine levels during NSA could have created a stronger association between the active lever and receiving a nicotine infusion, thus increasing the motivation to respond and resulting in the persistence of NSA behaviour in the absence of nicotine. Nicotine dose-dependently increases dopamine release in the nucleus accumbens (Di Chiara and Imperato, 1988; Marshall et al.,

1997), whereby dopamine release is thought to assign value to the behaviour associated with receiving reward (Ikemoto and Panksepp, 1999). Higher apparent doses of nicotine with inhibitor ICV treatment could lead to greater dopamine release in the nucleus accumbens which could increase the salience of lever pressing for nicotine.

There was no difference in responding follow priming nicotine injections by C8X ICV-treated rats during reinstatement. In both ICV treatments, animals did not reinstate NSA behaviour at any of the doses tested with IV nicotine injections but did reinstate NSA behaviour at the two highest doses with SC nicotine injection. This suggests that the nicotine doses given IV were not strong enough to reinforce active lever pressing, and in addition to the lack of dose-dependent increases in behaviour with this priming method it may explain why we do not see an effect of C8X. NSA behaviour was reinstated with the two highest SC priming doses, but there was no difference in responding between vehicle and inhibitor ICV-treated groups. These results suggest that the effect of C8X ICV treatment on central nicotine metabolism may not influence behavior sufficiently in this aspect of the model, despite significantly influencing motivation in PR and responding during extinction.

It is possible that the effects of C8X on NSA could be due an enhancement of the nicotine associated cues, such as the tone or light, either by C8X increasing cue reinforcement through nicotine's reinforcing effect on cues or on its own. We cannot distinguish between the effects of C8X on nicotine primary reinforcement vs. its effects on secondary cue reinforcement in our studies because cue reinstatement alone was not tested; however, if C8X increased nicotine-mediated cue reinforcement, it would still be consistent with its effect of increasing nicotine's reinforcing effect in NSA. If C8X ICV treatment increased cue reinforcement independent of nicotine, we would expect animals to increase NSA responding at all nicotine doses when given the inhibitor but this is not observed in acquisition or reinstatement. Thus, the effects of C8X ICV treatment were seen at specific doses, consistent with the hypothesized effect of brain CYP2B inhibition on nicotine reinforcement, suggesting that C8X did not increase the reinforcing effect of the cues independent of nicotine.

2.4 Relevance to human smoking

The effect of C8X ICV treatment on NSA behaviour in rats is consistent with the observed behavioural differences in smoking between *CYP2B6* slow and normal metabolizers. Preliminary

findings in one Caucasian adolescent population found that *CYP2B6* slow metabolizers converted to nicotine dependence faster when compared to normal metabolizers (Hoffmann, 2006). Like NSA acquisition, smoking acquisition in humans is a stage where sporadic nicotine exposure occurs (Wellman et al., 2004). Therefore, lower brain CYP2B6 levels in genetic slow metabolizers might reduce nicotine metabolism in the brain, leading to greater brain nicotine levels following each cigarette puff. This could make the experience of smoking cigarettes more reinforcing during the initial periods of smoking, which could explain the faster transition to nicotine dependence in adolescent *CYP2B6* slow metabolizers. It would also be analogous to greater acquisition of NSA with C8X ICV treatment in the rat, whereby inhibiting brain nicotine metabolism might have increased brain nicotine levels and subsequent nicotine reinforcement.

C8X ICV treatment did not influence NSA maintenance behavior (i.e. responding under FR schedule). Similarly, the number of cigarettes per day reported in smokers who are *CYP2B6* slow metabolizers did not differ from smokers who are *CYP2B6* normal metabolizers (performed at baseline in one smoking cessation study) (Lee et al., 2007b), suggesting that the intake behaviour of slow metabolizers while smoking is not different from normal metabolizers. This suggests that brain CYP2B activity can contribute to nicotine reinforcement when brain nicotine levels are transient, like that in acquisition, but not when nicotine intake is at a stable level, like in maintenance where the activity of hepatic CYP2A6 is primarily responsible for nicotine metabolism (Nakajima et al., 1996b). *CYP2B6* slow and normal metabolizers have similar plasma nicotine and cotinine levels with nicotine patch treatment, indicating that peripheral nicotine metabolism is not influenced by *CYP2B6* variation in the liver (Lee et al., 2007b). In support of this, CYP2B6 was found to contribute little to nicotine metabolism compared to CYP2A6 in human liver microsomes (Al Koudsi and Tyndale, 2010). Therefore, when *CYP2B6* slow metabolizers are current smokers their *CYP2B6* genotype is not likely to influence smoking behaviour via altered peripheral nicotine metabolism. Animals given C8X ICV also do not show differences in plasma nicotine and cotinine levels, suggesting that the inhibitor's effects on NSA behaviour were due its action in the brain. The consistency between humans and the animal model supports the idea that reduced brain CYP2B6 activity in CYP2B6 slow metabolizers could be responsible for the greater acquisition observed in adolescents and lack of differences reported in smoking behaviour.

Motivation and relapse, modeled by the PR and extinction and reinstatement paradigms, was increased by C8X ICV treatment. These findings in the rodent model were similar to the differences in these behaviours reported between slow and normal *CYP2B6* metabolizers. In the placebo arm of a bupropion smoking cessation trial, *CYP2B6* slow metabolizers experienced greater cravings during smoking abstinence (Lerman et al., 2002) and were more likely to relapse (Lerman et al., 2002; Lee et al., 2007a). The presence of these behavioural differences in cessation within the control group suggests that they were due to *CYP2B6* genotype. Greater brain nicotine levels in *CYP2B6* slow metabolizers during smoking could explain increases in motivation to smoke, leading to greater cravings and subsequent relapse. Greater brain nicotine would increase dopamine release, known to increase the salience of behaviours associated with the reward (Ikemoto and Panksepp, 1999), and thereby increase reinforcement of smoking behaviour. Thus, if smoking behaviour is more reinforced in *CYP2B6* slow metabolizers, then the behaviour could be harder to stop when these individuals attempt to quit.

Reduced brain CYP2B metabolism could influence the selection of smoking cessation treatments, such as bupropion, because it is a CYP2B substrate (Hesse, 2000). Bupropion does not have an effect on CYP2B mRNA, protein or activity in human hepatocytes (Hesse et al., 2003), suggesting that continued treatment does not influence bupropion metabolism. However, *CYP2B6* slow metabolizers produce less hydroxybupropion, the main metabolite formed from bupropion hydroxylation (Schroeder, 1983), demonstrating that reduced CYP2B activity can reduce bupropion metabolism (Zhu et al., 2012). Zhu et al. (2012) found that hydroxybupropion, and not bupropion, plasma levels correlated with cessation outcome, suggesting that this metabolite mediates bupropion's effect on smoking. Furthermore, *CYP2B6* genotype was significantly associated with hydroxybupropion formation (Zhu et al., 2012), indicating that *CYP2B6* genotype could alter cessation outcome by this treatment. CYP2B6 variation in the liver mediates this effect; however slower metabolism by brain CYP2B6 would also reduce metabolism of bupropion to hydroxybupropion and contribute to lower hydroxybupropion levels in the brain where the metabolite might exert its effect on cessation (Damaj et al., 2004). To date, bupropion metabolism in the brain has not been investigated, however our group has previously demonstrated that inhibiting CYP2B activity in the brain can increase brain propofol (Khokhar, 2011) and chlorpyrifos levels (Khokhar, 2012) and the work described here demonstrated that inhibiting CYP2B activity can increase brain nicotine levels. Thus, this data suggests that

CYP2B6 variation in the brain could also alter brain bupropion and hydroxybupropion levels. This could be tested by inhibiting CYP2B in the brain with C8X and measuring brain bupropion levels in our brain microdialysis protocol.

The role of hydroxybupropion in bupropion-mediated cessation is still not clear, as individuals with *CYP2B6*4* allele, which increases bupropion metabolism and subsequently increases hydroxybupropion formation (Kirchheiner et al., 2003), report lower success rates with bupropion treatment (Tomaz et al., 2015) in contrast to previous findings of lower hydroxybupropion to bupropion (i.e. slower metabolism) being associated with lower success rates (Zhu et al., 2012). Further investigation of the mechanisms by which hydroxybupropion might contribute to smoking cessation might clarify these discrepancies between *CYP2B6* genetic variation and bupropion-mediated cessation. One approach would be to investigate the effects of bupropion vs. hydroxybupropion in NSA extinction and reinstatement, the animal models of cessation and relapse. Bupropion is converted to hydroxybupropion to a lesser extent in the rat compared to humans and mice (Suckow et al., 1986; Welch et al., 1987), and along with enzymatic inhibitor approaches, these NSA experiments could provide a means to test the effects of the parent and metabolite separately and combined. This could assist in elucidating whether the effects on smoking cessation are due to the metabolite alone, or a combination of the parent and metabolite.

2.5 Future behavioural experiments using brain CYP2B inhibition

Inhibition of brain CYP2B activity was investigated in the limited access NSA paradigm however extended and unlimited access paradigms also exist. Limited access is short periods of time (one to three hour sessions) where animals can receive drug infusions contingent upon the behavioural response; thereby animals learn that this is the time period of drug availability and respond for the desired amount of drug (Matta et al., 2007). With limited access, the primary reinforcing effect of nicotine doses can be measured through changes in responding within the same amount of time with changing nicotine infusion dose, as displayed in nicotine's dose-response curve (Corrigall and Coen, 1989; Donny et al., 1995). This results in the formation of stable patterns of responding and nicotine intake by nicotine dose, whereby factors that influence nicotine's primary reinforcing effect can be investigated. Therefore, in our first investigation of

altering brain CYP2B activity, changes in the dose-dependent reinforcing effect of nicotine could be tested with the limited access paradigm.

Extended and unlimited access paradigms provide different information about nicotine reinforcement than limited access. Extended access is longer periods of time (four to twelve hour sessions) where animals can receive drug infusions contingent upon the behavioural response (Paterson and Markou, 2004) and unlimited access is 24 hour access to receive drug infusions (Valentine et al., 1997), which can reveal patterns of drug intake throughout the day. If C8X ICV injection increases brain nicotine levels following initial nicotine infusions than greater responding would be expected in the first few hours of the first few sessions during acquisition, consistent with that seen in our limited access paradigm. We did not observe an effect of C8X ICV treatment on responding during FR maintenance with limited access, therefore it would be interesting to investigate whether C8X ICV treatment would alter the pattern of responding with longer access periods. During maintenance, where stable responding should occur, C8X ICV treatment would not be expected to alter total responding, however there might be periods of time in which increased brain nicotine levels may influence responding: dose-dependent decreases in responding have been observed in specific time periods during the dark phase in unlimited NSA (Valentine et al., 1997), suggesting the possibility that responding during specific times could be altered with brain CYP2B inhibition without affecting total responding.

Nicotine dependence, measured by somatic signs in the rat model of nicotine withdrawal (Malin et al., 1992), can be established in these longer access schedules (Paterson and Markou, 2004; Matta et al., 2007). Nicotine withdrawal can be provoked spontaneously, by the removal of nicotine infusions, or precipitated by mecamylamine treatment (Malin et al., 1994), which antagonizes nicotinic receptors in the brain and thus prevents nicotine's actions (Martin et al., 1989). Mecamylamine-precipitated withdrawal is also observed in limited access; however, the withdrawal is not as persistent as that seen with longer access schedules. Withdrawal can be observed up to two weeks after abstinence from limited access NSA while withdrawal signs are still observed up to four weeks after abstinence from extended access NSA (six hour session) (Paterson and Markou, 2004), suggesting that longer-term nicotine dependence can be achieved with longer access to NSA. Therefore, extended or unlimited daily access would be a better variation of the NSA model to test the effect of C8X ICV treatment on nicotine dependence, as measured through nicotine withdrawal signs. C8X ICV treatment increased motivation and

persistence in responding during limited access NSA; this would also be expected in longer access schedules, where stronger associations between responding and drug reward might elicit greater or more persistent withdrawal signs.

Other rodent models of drug reward could confirm the effect of brain CYP2B inhibition on nicotine-mediated behaviours. Intra-cranial self-stimulation (ICSS) and CPP are two models that test whether a compound is rewarding (Tzschentke, 1998; Stoker and Markou, 2011); both have demonstrated that nicotine has rewarding properties (Fudala et al., 1985; Huston-Lyons and Kornetsky, 1992). Drugs of abuse reduce the threshold for responding in ICSS, which is defined as the lowest level of electrical stimulation that rats will respond to in order to stimulate the given brain area (Stoker and Markou, 2011). Nicotine was shown to reduce ICSS threshold at multiple doses in the medial forebrain bundle, the collection of tracts that include connections from the ventral tegmental area, which was blocked by mecamylamine treatment (Huston-Lyons and Kornetsky, 1992). This effect in the same region was also found in more recent studies with similar nicotine doses (Harrison et al., 2002; Harris et al., 2015). Using the ICSS model, where nicotine dose-dependently lowered ICSS threshold, C8X ICV treatment would be expected to increase the reduction in threshold at lower nicotine doses, due to increasing the apparent dose. Conditioned place preference typically utilizes a two chamber operant box, one with a preferred environment (e.g. Dark walls, mesh floor) for rats and one with a non-preferred environment (e.g. Light walls, bar floor), where SC injections of the test drug are paired with the non-preferred chamber and saline injections are paired with the preferred chamber in multiple sessions in order for the association between drug and chamber to develop (Tzschentke, 1998). Drugs are considered rewarding when animals, not given any SC injection and allowed to move freely between both chambers, choose to stay in the non-preferred chamber. Because animals are willing to stay in the chamber that they did not prefer before exposure to the drug, it suggests that the rewarding effect of the drug took precedence over any unpleasantness from the non-preferred environment (Bardo and Bevins, 2000). Nicotine can dose-dependently increase time spent in the non-preferred environment, first demonstrated by Fudala et al. (1985) and subsequently repeated in numerous studies reviewed by Le Foll and Goldberg (2005). C8X ICV treatment can increase brain nicotine levels following an acute injection of nicotine SC; therefore we would expect C8X ICV treatment to increase time spent in the nicotine-associated chamber in

line with a perceived higher dose of nicotine. These experiments would provide further evidence to support that reducing brain CYP2B activity could significantly influence nicotine reward.

3 Effect of altering brain CYP2B activity on nicotine levels in the brain

3.1 Brain CYP2B inhibition in nicotine microdialysis

C8X ICV treatment prior to nicotine administration resulted in higher brain nicotine levels compared to vehicle ICV treatment. This was found after nicotine was administered IV and SC, demonstrating that C8X can increase nicotine levels in the brain following peripheral injections regardless of the route of administration. Higher brain levels of nicotine with inhibitor treatment would be consistent with greater nicotine reinforcement following nicotine infusions in NSA and explain the differences in acquisition, PR, and nicotine-seeking behaviour between rats treated with inhibitor vs. vehicle.

Higher brain nicotine levels could increase the primary rewarding effects of nicotine, which would explain the increase in NSA responding during initial nicotine exposure with inhibitor treatment, and enhance or facilitate changes in the circuitry involved in developing dependence, which would explain the increase in motivation and persistence in NSA responding in the absence of nicotine. The primary rewarding effect of nicotine is thought to be mediated through dopamine release in the nucleus accumbens; this has been demonstrated in rat brain through microdialysis (Di Chiara and Imperato, 1988) and in human smokers through PET imaging (Brody et al., 2004; Brody et al., 2008), although increases in dopamine release in humans have not been consistently found (Barrett et al., 2004; Montgomery et al., 2007). Dopamine release in response to rewarding stimuli occurs through burst firing or phasic activation of VTA neurons (Schultz, 2002); using guinea pig striatal slices, nicotine was found to activate nAChRs on VTA neurons to elicit phasic activation (Rice and Cragg, 2004). Nicotine also enhanced phasic activation while suppressing tonic activation, which is single background spike activity (Grace and Bunney, 1984).

This transition from tonic to phasic activity by nicotine might be through desensitization of nAChRs, as blocking receptor activity through mecamylamine enhanced dopamine release in response to reward-related electrical stimulation in a similar manner as nicotine treatment (Rice and Cragg, 2004). Acute nicotine desensitizes nAChRs on VTA neurons (Pidoplichko et al., 1997), which would be expected to reduce VTA-mediated dopamine release. However, nAChRs are expressed in glutamatergic and GABAergic neurons that have synaptic inputs to the VTA (Kalivas et al., 1993; Mansvelder and McGehee, 2000; Mansvelder et al., 2002), implicating the involvement of these systems in enhanced dopamine signaling. Antagonists of NMDA receptors can reduce nicotine-induced dopamine release from the VTA (Schilstrom et al., 1998), suggesting that glutamate signaling can modulate this process. This might occur through nicotine activating nAChRs on glutamate projections, because nicotine can produce long term potentiation (LTP) in glutamatergic input from the prefrontal cortex to the VTA in rat brain slices (Mansvelder and McGehee, 2000). Moreover, NMDA receptors have been shown to mediate VTA dopamine burst firing (Murase et al., 1993), providing further evidence to suggest that nAChR-mediated glutamate release in the VTA is involved in its potentiation of phasic activation. This glutamatergic LTP in the VTA was dose-dependent, as higher nicotine doses increased the rate of spontaneous excitatory postsynaptic currents (EPSCs) (Mansvelder and McGehee, 2000). Therefore, greater nicotine levels in the brain could increase VTA dopamine firing.

GABA interneurons within the VTA (Margolis et al., 2012) and reciprocal GABAergic projections both to (Kalivas et al., 1993) and from (Van Bockstaele and Pickel, 1995) the nucleus accumbens and VTA express nAChRs (Klink et al., 2001), which suggests that these may be the sites where nAChR desensitization mediates changes in dopamine signaling. Application of nicotine, in concentrations similar to that of plasma levels in smokers, to rat brain slices resulted in initial increases in GABAergic inhibitory postsynaptic currents (IPSCs) that reduced over time until it was abolished (Mansvelder et al., 2002), suggesting that nAChRs on GABA inputs were subsequently desensitized. This desensitization was dose-dependent as higher doses of nicotine pre-treated to slices displayed greater reductions in the frequency of IPSCs (Mansvelder et al., 2002). A recent study using *in vivo* electrophysiology in mice demonstrated that initial GABA interneuron activation by nicotine may be involved in phasic dopamine release but that repeated activation of these neurons by nicotine leads to nAChR desensitization, resulting in prolonged

elevation of dopamine (Tolu et al., 2013). This difference in GABAergic activity after single versus repeated nicotine exposure suggests that reducing GABA-mediated inhibition in the VTA may be a consequence of continued nicotine intake. Furthermore, GABA interneuron activation was mediated by β_2-containing receptors, as β_2-knockout mice only show this increase in phasic dopamine release by nicotine when β_2 is re-expressed by lentiviral delivery (Tolu et al., 2013). Only one nicotine dose was tested in these experiments, therefore it would be interesting to test whether dose-dependent increases in nicotine with repeated exposure could increase desensitization and reduce GABAergic inhibition.

The cellular architecture of the VTA is heterogeneous, containing dopamine, GABA and glutamate neurons with various connections to other brain regions (Lammel et al., 2014), which adds complexity to the effects of nicotine in brain reward. Therefore, it is possible that changes in brain nicotine levels, such as increased nicotine by reduced metabolism in the brain, could contribute to changes in dopamine firing and nAChR activation and desensitization by nicotine, leading to increased salience of reward-related stimuli. This would be consistent with the increase in NSA acquisition seen with CYP2B inhibition at the 7.5 μg/kg dose of nicotine, where inhibition may increase brain nicotine levels sufficient enough to activate glutamatergic and/or GABAergic inputs to the VTA and result in greater dopamine release. This would increase the rewarding effect of this low nicotine dose which, under normal conditions, would produce lower brain nicotine levels. The nAChR subtypes expressed in these inputs can differ (Mansvelder et al., 2002), with α7-containing receptors found on glutamatergic inputs (Mansvelder and McGehee, 2000) and α4β2-containing receptors found on GABAergic interneurons (Mansvelder et al., 2002; Tolu et al., 2013). Given that these nAChR subtypes have different sensitivity to nicotine (Wooltorton et al., 2003), it is possible that increases in brain nicotine levels could alter the activation and desensitization of these receptors in a cell type-specific manner that increases dopamine release and subsequent nicotine reinforcement.

Brain CYP2B inhibition by C8X would be expected to reduce brain levels of nicotine metabolites formed by CYP2B, such as cotinine and nornicotine (Hammond et al., 1991; Yamanaka et al., 2005); however, this was not determinable because cotinine levels were low or near the limit of detection and nornicotine was below the limit of detection. Other nicotine metabolites, such as nicotine-1'-*N*-oxide, 3-hydroxycotinine, and norcotinine were also below the limit of detection. Consistent with this, previous nicotine microdialysis studies found low

(Woods et al., 2006) or undetectable (Chang et al., 2005) levels of cotinine in the brain after IV nicotine (the other nicotine metabolites were not investigated by microdialysis in these studies). Cotinine, nornicotine, norcotinine and nicotine-1'-*N*-oxide have been detected in rat brain homogenate (Ghosheh et al., 1999; Ghosheh et al., 2001; Craig et al., 2014) while 3-hydroxycotinine was measured but not detected in Craig et al. (2014), suggesting that nicotine metabolites can be found in the brain. It is possible that, because extracellular levels of these metabolites from the cerebrospinal fluid (CSF) are collected with microdialysis as opposed to whole tissue levels with brain homogenate, these metabolites are not present in the CSF in appreciable amounts.

3.2 Brain CYP2B induction in nicotine microdialysis

Brain CYP2B induction by 7-day nicotine treatment reduced brain nicotine levels measured by microdialysis. This was tested within-animal, demonstrating that rats repeatedly treated with nicotine display lower brain nicotine levels compared to the first injection of nicotine. 7-day, but not 1-day, nicotine treatment has been shown to induce CYP2B mRNA and protein in the brain of rats (Miksys et al., 2000a; Khokhar, 2010); the current findings using this treatment paradigm suggest that brain CYP2B was induced in these rats, leading to increased nicotine metabolism and lower brain nicotine levels. Lower brain nicotine levels following systemic nicotine administration could reduce the reinforcing effect of nicotine; this could lead to reductions in nicotine-mediated behaviour if its association with nicotine reward has not been established, such as in acquisition. Exposure to CYP2B inducers, such as phenobarbital (Schilter and Omiecinski, 1993; Lee et al., 2006b), that can increase brain CYP2B activity before nicotine administration might then reduce nicotine-mediated behaviour. Conversely, lower brain nicotine levels could contribute to continuation of nicotine-seeking behaviour after nicotine reinforcement has already been established, in order to receive the rewarding effect associated with the behaviour.

Higher CYP2B6 protein levels have been reported in the post-mortem brains of human smokers (Miksys, 2003), which is consistent with brain CYP2B induction after repeated nicotine exposure in rats (Miksys et al., 2000a; Khokhar, 2010) and monkeys (Lee et al., 2006a; Ferguson et al., 2013), suggesting that CYP2B6 could be induced by cigarette smoking. This also suggests that CYP2B induction in the rat brain might be possible in the animals that self-administered

nicotine. The total nicotine intake during self-administration may reach the same levels of the nicotine dose used in 7-day nicotine treatment, as animals that self-administered the 7.5 µg/kg dose averaged 0.3 mg/kg nicotine per session in FR2 and CYP2B protein induction can be induced with 7-day treatment using 0.1-1.0 mg/kg nicotine (Miksys et al., 2000a). However, the dosing schedule of nicotine administration was different in NSA compared to the 7-day nicotine treatment, where animals receive this total dose of 0.3 mg/kg nicotine over the 2 hour NSA session by repeated infusions of a smaller nicotine dose compared to the single bolus injection of nicotine in 7-day nicotine treatment. It is not known whether the induction is due to repeated nicotine exposure by daily treatment (duration of nicotine exposure) or due to repeated nicotine exposure of a bolus nicotine injection (combination of duration and dose), and would therefore need to be tested. CYP2B expression in the brain following NSA has not yet been investigated but could be examined using a group of animals that are saline-yoked during FR self-administration. Saline-yoking involves pairing an animal that receives nicotine infusions with another animal, where the second animal receives a saline infusion at the same time the first animal receives nicotine. To examine whether CYP2B expression is increased after NSA, these animal pairs can undergo NSA acquisition and maintenance using the FR schedule. Thus, the exposure to IV infusions from NSA can be controlled for and any effects on CYP2B expression can be attributed to nicotine. At the end of NSA, CYP2B mRNA and protein levels in the brain and liver can be measured in the animals that acquired NSA compared to their saline-yoked counterparts. *In vivo* nicotine microdialysis could also be conducted, in another set of animals, to determine whether lower brain nicotine levels are observed following a single nicotine injection.

If CYP2B inhibition in the brain reduces nicotine metabolism and increases brain nicotine levels, which would increase nicotine reinforcement, then it would be expected that inhibitor treatment would increase NSA responding compared to vehicle. There was no effect of inhibitor on NSA under the FR schedule after acquisition, which could be due to the lack of dose-dependent changes in responding under FR2. However, an alternative explanation could be that CYP2B induction in the brain from NSA sessions increased responding in the vehicle treated animals. In vehicle treated animals, greater CYP2B activity from induction might have increased responding to obtain desired nicotine levels, while CYP2B induction in inhibitor treated animals would not alter responding because inhibitor treatment would inhibit induced CYP2B. Therefore, this

increase in responding may be due to the change in reinforcing effect of nicotine following induction in vehicle treated animals which would not occur in animals given inhibitor.

The effect of brain CYP2B induction on the PR schedule would be difficult to determine because animals are still receiving nicotine infusions, albeit at a lower total level of nicotine (0.05-0.2 mg/kg) compared to the FR schedule (0.2-0.6 mg/kg). When animals switch to the PR schedule a drop in responding occurs in the first session and then increases as sessions continue until they reach relatively stable levels of responding (Donny et al., 1999). Brain CYP2B levels are induced up to 24 hours after the last nicotine injection and can return to baseline levels seven days thereafter in the absence of nicotine (Khokhar, 2010). In the PR schedule animals are still given nicotine, therefore if CYP2B induction can occur during FR, it is possible that brain CYP2B levels may remain induced during the PR schedule. It is currently unknown whether continued exposure to lower levels of nicotine, then that given under the 7-day paradigm or the FR schedule, would reduce or maintain levels of induced CYP2B. For the 7-day nicotine injection paradigm, this could be tested by examining brain CYP2B mRNA and protein levels in animals given extra injections of lower nicotine doses after the seventh nicotine injection. For animals that undergo NSA, brain CYP2B induction after FR maintenance would first be determined by *in vivo* nicotine microdialysis after the last FR session using nicotine and saline-yoked animal pairs. If brain CYP2B is induced then brain nicotine levels are expected to be lower in animals receiving nicotine compared to animals receiving saline. These animal pairs would then undergo PR NSA, where animals given nicotine could be further split into one group still receiving nicotine and the other group receiving saline. Brain CYP2B levels in animals that received nicotine during FR but saline during PR could be compared to the animals that received nicotine throughout FR and PR, to determine whether the nicotine levels obtained from PR NSA were sufficient enough to maintain induction of brain CYP2B levels.

The effect of the inhibitor on the PR schedule would not be expected to be influenced by enzyme induction in the brain because animals received inhibitor after animals established a baseline level of PR responding. Thus at the time of vehicle ICV injection, which did not alter baseline responding, nicotine exposure is stable, suggesting that CYP2B activity has not changed between PR sessions. The subsequent changes in responding reported after inhibitor ICV treatment would then be attributed to inhibition of this level of CYP2B activity.

CYP2B induction in the brain could be one of the consequences of long-term nicotine exposure. With repeated nicotine exposure, further changes have been reported in nAChRs expression and activity in mesolimbic regions (Picciotto et al., 2008; D'Souza and Markou, 2011), possibly through compensatory adaptations to re-establish homeostasis. In nAChR-containing cell lines chronically treated with nicotine, prolonged nAChR desensitization results in up-regulation of various nAChRs subtypes, including $\alpha_4\beta_2$ and α_3- and α_7-containing receptors (Peng et al., 1997; Fenster et al., 1999), although the latter two subtypes are up-regulated with higher nicotine concentrations than that obtained from smoking. Up-regulated receptors are active, as increased channel efflux correlate with increased receptor binding in rat brain (Nguyen et al., 2004), which could alter neuronal activity of the circuits these receptors are expressed within. Increased nAChR binding is observed in the brains of rats that self-administered nicotine (Donny et al., 2004) and in the post-mortem brains of human smokers (Benwell et al., 1988; Breese et al., 1997), consistent with long-term exposure to nicotine altering nAChR architecture and activity. Therefore, repeated nicotine treatment would lead to nicotine accumulation in the brain, whereby CYP2B induction in the brain might increase CYP2B activity and lead to decreasing nicotine levels.

Neuroadaptations from chronic nicotine exposure are thought to contribute to the withdrawal syndrome that smokers experience in the absence of nicotine (De Biasi and Salas, 2008; De Biasi and Dani, 2011). These changes in neurocircuitry were established to accommodate the repeated presence of nicotine in the brain; however, without nicotine these changes are not necessary and thus may lead to maladaptive symptoms that can reinforce nicotine-seeking behaviour in order to reduce these symptoms (Koob et al., 2004). In humans, nicotine withdrawal syndrome consists of somatic and negative affective symptoms including nicotine cravings, increased appetite, weight gain, decreased heart rate, sleep disturbances, irritability, anxiety, depressed mood and problems concentrating (Hughes and Hatsukami, 1986; Hughes, 2007). These symptoms can arise as early as four hours after the last cigarette (Piasecki et al., 2003; Hughes, 2007; De Biasi and Salas, 2008), suggesting that the precipitation of these symptoms following withdrawal from nicotine may then maintain smoking behaviour in order reduce them. Thus, while smoking behaviour may be initially driven by the rewarding effects of nicotine, the persistence in smoking behaviour after changes in reward neurocircuitry may then also be driven by the negative reinforcing effects of withdrawal from nicotine. During abstinence these symptoms can persist

for weeks after cessation (Piasecki et al., 2003; Hughes, 2007), suggesting that they can also contribute to relapse (Baker et al., 2004).

Somatic and affective symptoms can also be observed in the rodent model of nicotine withdrawal characterized by Malin et al. (1992). Somatic signs in the rat included gasping, tremors, shakes, teeth chattering and vacuous chewing movement which was precipitated dose-dependently by nicotine withdrawal. Nicotine re-administration reduced these behaviours, suggesting that they were brought about by nicotine cessation (Malin et al., 1992). Induced brain CYP2B in nicotine dependent humans or rodent would be expected to reduce brain nicotine levels during smoking, which would reduce the alleviation of early nicotine withdrawal and possibly drive continued smoking behaviour; however, in smoking cessation lower brain levels of nicotine following nicotine relapse would reduce the reinforcing effect of nicotine and possibly reduce positive reinforcement of smoking behaviour. Although negative reinforcement can contribute to nicotine relapse (Baker et al., 2004), positive reinforcement has also been shown to predict relapse to smoking (Strong et al., 2011). Therefore, it is possible that if brain CYP2B is induced during cessation it could reduce the positive reinforcing effect of nicotine and aid abstinence. Brain CYP2B induction in rats returns to baseline levels a week after the last nicotine treatment (Khokhar, 2010), suggesting that greater CYP2B activity in early abstinence but lower CYP2B activity in continued abstinence might contribute to nicotine relapse.

CYP2B induction is only one of many changes that result from chronic nicotine treatment (Konu et al., 2001; Li et al., 2004) and other CYP enzymes, such as CYP2D in the brain, are also induced by this paradigm (Yue, 2008). To confirm that the changes in brain nicotine levels were due to CYP2B induction in the rat brain, the CYP2B inhibitor C8X was used. C8X is considered a selective inactivator for CYP2B1 because although C8X can inactivate CYP2E1, this occurs at a 25-fold higher concentration compared to CYP2B1 (Yanev, 1999). The mechanism of CYP2E1 inactivation by C8X is not known, however CYP2E1 does not metabolize nicotine (Nakayama et al., 1993) so it is not expected to influence nicotine levels in the brain. C8X can also weakly inhibit, but not inactivate, CYP2D6 (Yanev, 1999) which has low catalytic activity towards nicotine (Nakayama et al., 1993). Thus, given that C8X ICV injections are administered 22 hours prior to testing its effect on nicotine; it is unlikely that C8X inhibited CYP2D in the brain at the time of testing as competitive inhibition of CYP2D by C8X would be unlikely to occur after this length of time. ICV treatment of C8X increased brain nicotine levels in the acute condition,

where animals were nicotine naïve, suggesting that C8X inhibited brain CYP2B activity and reduced nicotine metabolism. The effect of C8X on brain nicotine levels over time was consistent with the effect of increasing the nicotine dose of the SC injection, specifically brain nicotine levels were higher with C8X ICV treatment at the time points where a dose-dependent difference in nicotine levels were observed. This is also consistent with the effect of C8X ICV treatment on brain nicotine levels after an IV injection, where increases in brain nicotine levels with C8X ICV treatment were seen during time points that displayed dose-dependent increases in nicotine levels, and indicates that these changes are specific to nicotine pharmacokinetics and not due to indirect effects of inhibiting other CYP enzymes, such as CYP2E1. C8X ICV injections throughout and after 7-day nicotine treatment also increased brain nicotine levels, indicating that C8X was able to inhibit induced CYP2B and also suggesting that it was indeed induced CYP2B, versus another enzyme, that was responsible for the reduction in brain nicotine levels following 7-day nicotine treatment. These experiments using the CYP2B inhibitor demonstrate that compounds that can alter brain CYP2B activity can influence brain nicotine levels, suggesting that exposure to inducers or inhibitors could change brain CYP2B activity enough to alter the brain levels of its substrates.

The nicotine metabolites, like that observed with IV nicotine injections, were low or below the level of detection. Brain cotinine levels were higher with SC injection than IV, consistent with the higher nicotine dose used for SC injection (1 mg/kg) vs. IV (0.15 mg/kg); however, no difference was observed in the low levels of brain cotinine between acute and chronic microdialysis sessions. Similar to the explanation for IV nicotine microdialysis, it is possible that the methods we use for microdialysis are not sensitive enough to detect changes in metabolite levels.

3.3 Future experiments manipulating brain CYP2B activity

Our nicotine microdialysis experiments did not include the assessment of nicotine-mediated behaviours. Withdrawal signs after 7-day nicotine treatment could also be tested in conjunction with collecting brain dialysate to determine if these symptoms can be precipitated after this pattern of nicotine exposure. This would suggest that CYP2B could be induced at the beginning of nicotine withdrawal, which could lead to a reduction in nicotine levels if the animal is then exposed to nicotine. Lower nicotine levels in the brain during withdrawal could be insufficient to

reduce withdrawal symptoms if neuroadaptative changes have manifested, such as increases in nAChR expression, which might lead to increases in nicotine-seeking behaviour.

The effect of brain CYP2B induction on NSA acquisition could also be examined. This was not tested in our NSA experiments because our goal was to test whether variation in brain CYP2B activity could influence NSA behaviour; therefore, blocking CYP2B activity in the brain would demonstrate whether it modulates this outcome. Brain CYP2B inhibition was also used in NSA to model slow metabolism by *CYP2B6* genetic variation, which would support the idea that reduced metabolism of nicotine by CYP2B6 in the brain may influence smoking behaviours. Therefore, now that we have established that CYP2B activity can play a role in NSA, the role of CYP2B induction could also be explored.

Repeated nicotine treatment before NSA has been tested using a paradigm similar to the 7-day nicotine treatment used in CYP2B induction. This paradigm consisted of seven daily injections of 0.4 mg/kg/SC nicotine prior to the start of NSA (Shoaib et al., 1997). Nicotine pre-treatment altered acquisition in a strain-specific manner: Sprague Dawley rats showed greater acquisition while Long-Evans rats showed lower acquisition (Shoaib et al., 1997). CYP2B activity was not investigated in this study so it is unknown if these differences were due to differential induction of brain CYP2B or to effects independent of CYP2B. Rat strain differences exist in phenobarbital induction of liver CYP2B (Larsen and Jefcoate, 1995), but species-specific induction of brain CYP2B has not been investigated. One approach to test if induced brain CYP2B was involved in the observations made by Shoaib et al. (1997), would be to inhibit brain CYP during SC pretreatment. Within each rat strain, rats would receive either saline or nicotine SC treatment and within each saline or nicotine group, half of the group would receive ICV injections of vehicle (ACSF) or the CYP2B inhibitor (C8X) 22 hours prior to the SC injection.

We could test acquisition in nicotine naïve rats using a different inducer, such as phenobarbital. PB can induce brain and liver CYP2B (Schilter et al., 2000), but it might be possible to select a dose and duration where PB given ICV could selectively induce CYP2B in the brain. Brain and liver CYP2B can be induced by giving rats four intraperitoneal injections of PB (Schilter et al., 2000), so this paradigm could be tested for brain selective induction of CYP2B using ICV injections of PB. Once a protocol for PB induction with ICV injection is established, NSA acquisition could then be carried out. Induction of brain CYP2B would be expected to increase

nicotine metabolism in the brain, which would decrease nicotine levels following nicotine infusion and reduce the reinforcing effect of nicotine. Lower NSA acquisition would then be anticipated with brain CYP2B induction.

The levels of brain CYP2B could also be examined in animals that have received alternate forms of chronic nicotine treatment, such as extended access NSA, to investigate whether brain CYP2B can be induced with volitional long-term nicotine intake. To test this, rats would acquire NSA on an extended access schedule and following stable maintenance they would be sacrificed and their brains removed to measure CYP2B protein levels by western blotting as previously characterized (Miksys et al., 2000a; Khokhar, 2010) in comparison to saline-yoked animals. Because brain CYP2B induction has only been reported following bolus SC injections of nicotine, given once a day, at doses as low as 0.1 mg/kg (Miksys et al., 2000a), a range of nicotine doses by which NSA is established under extended access would be used to determine the extent of induction by dose. O'Dell et al. (2007) established NSA with a 23-hour access schedule using 0.015, 0.03, and 0.06 mg/kg/IV doses where intake increased dose-dependently, therefore brain CYP2B induction could be measured after NSA with this dose range using microdialysis after a single nicotine injection. Specificity of induction for CYP2B could be tested with C8X ICV treatment, where a single C8X ICV pretreatment would be expected to reverse or reduce the effect of induced CYP2B on nicotine brain levels measured by microdialysis.

The microdialysis and NSA behaviour experiments utilized ICV injection of inhibitor, which would lead to whole brain CYP2B inhibition as the inhibitor would circulate throughout the brain by CSF flow. To determine if regional inhibition of CYP2B could influence local brain nicotine levels and nicotine-mediated behaviours, such as regions within the brain reward system, inhibitors could be injected IC. The first area to inject locally would be the VTA, where nAChR activation and desensitization is implicated in the rewarding effect of nicotine and is necessary for NSA in rats (Corrigall et al., 1992; Corrigall et al., 1994). CYP2B protein has not been specifically quantified in the VTA; however, CYP2B protein has been detected in the rat brain stem and substantia nigra (Miksys et al., 2000a), suggesting that CYP2B is expressed near this region. Therefore, the effect of C8X IC injection in the VTA on NSA acquisition and nicotine microdialysis could be tested to determine if local CYP2B inhibition could increase behaviour and local nicotine levels. Other regions that would be interesting to test CYP2B inhibition include the nucleus accumbens and frontal cortex: nAChRs expressed in these regions

are involved in mediating nicotine reinforcement (Feduccia et al., 2012) and of these regions CYP2B protein can be found (Miksys et al., 2000a). Regional inhibition of CYP2B is expected to reduce nicotine metabolism and increase the local levels of nicotine. Increases in local levels of nicotine in these regions might increase nAChR activation, thus increasing nicotine reinforcement and subsequent NSA behaviour.

In these current studies brain CYP2B activity was altered by pharmacological methods; however, molecular methods can also be used to alter CYP2B activity. CYP2B gene expression could be knocked down by injection small interfering or small hairpin ribonucleic acid (siRNA or shRNA) in the rat brain. This method was used to investigate the role of μ-opioid receptors in heroin-mediated behaviour, where siRNA targeting the μ-opioid receptor was injected into the substantia nigra and VTA in mice and resulted in reducing locomotor activity and CPP induced by heroin (Zhang et al., 2009). Rat CYP2B1 siRNA can be obtained and has been shown to silence Cyp2b1 gene expression in rat glomerular epithelial cells (Tian et al., 2010); therefore it can be tested to determine that CYP2B1 siRNA can reduce brain gene expression *in vivo* and if this reduction can alter nicotine-mediated behaviour. Viral vector delivery of the CYP2B gene into the brain, where a virus vector containing the gene enters the cell (Davidson and Breakefield, 2003), could be used to over-express CYP2B analgous to inducing brain CYP2B. Using lentiviral delivery, Fowler et al. (2011) was able to re-express α_5 receptors in the habenula of knockout mice to test its role in NSA. Another study was able to inject a viral vector containing the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subunit GluR1 into the rat VTA, which led to increases in morphine-induced locomotor activity and CPP (Carlezon et al., 1997). These studies suggest that viral vector delivery of the CYP2B gene is feasible in specific regions within the rat brain.

4 General Discussion

4.1 Role of brain CYP2B at the blood brain barrier

Brain CYP2B was inhibited throughout the brain and induction by nicotine can be observed in multiple regions in a cell-specific manner (Miksys et al., 2000a); therefore, we cannot distinguish whether altering brain CYP2B in one or multiple different regions is responsible for the changes

in brain nicotine levels. As nicotine is administered peripherally and needs to cross the blood-brain barrier in order to reach the brain, it is possible that altering CYP2B expressed at this barrier could contribute to changes in brain nicotine levels. CYP2B6 is expressed in human astrocytes (Miksys, 2003) and microvessels (Dauchy et al., 2009) present at the blood-brain barrier and CYP2B1 is constitutively expressed in rat endothelial cells and astrocytes that line blood vessels and the choroid plexis (Volk et al., 1995; Miksys et al., 2000a). CYP2B6 protein levels are higher in the astrocytes of post-mortem brain of human smokers compared to non-smokers (Miksys, 2003), suggesting that CYP2B induction by nicotine occurs at the blood-brain barrier.

Nicotine rapidly crosses the blood-brain barrier and enters the brain following peripheral nicotine administration (Hukkanen et al., 2005; Rose et al., 2010), where nicotine from a single puff of a cigarette can be detected after seven seconds (Rose et al., 2010). One PET study using ^{11}[C]-nicotine cigarettes reported that dependent smokers displayed slower brain up-take kinetics compared to non-dependent smokers, where dependent smokers showed slower brain accumulation of nicotine and lower maximal nicotine concentrations (Rose et al., 2010). Slower brain uptake was found in this study to be a consequence of, at least in part, slower nicotine removal from the lungs, measured by the changes in radioactivity in lungs and brain. This study demonstrated that chronic nicotine exposure could alter brain nicotine uptake.

Brain CYP2B induction at the blood-brain barrier could possibly contribute to lower brain nicotine level accumulation by metabolizing nicotine as it crosses into the brain. This could be tested using *in vitro* models of the blood brain barrier, where CYP2B can be pharmacologically induced in reconstituted endothelial cells from rat (Abbott et al., 2012) or human (Weksler et al., 2005). Nicotine flux from the theoretical blood partition to the theoretical brain partition could be measured, where nicotine levels in the brain partition would be expected to decrease with CYP2B induction. This could also be tested *in vivo* with specific over-expression of the CYP2B gene in blood-brain barrier cells. The Cre-loxP system involves the excision or insertion of genetic material contained within loxP sites by Cre recombinase, which allows for the knockout or over-expression of a gene where Cre recombinase is expressed (Michel et al., 2010). A mouse line with endothelial cell-specific expression of Cre recombinase has been characterized (Li et al., 2005), suggesting that specific over-expression of CYP2B in endothelial cells of the blood-brain barrier could be possible using this system. With the *in vivo* over-expression of CYP2B

specifically in blood-brain barrier cells, nicotine microdialysis could be conducted to measure nicotine levels that have crossed into the brain.

4.2 Brain CYP2B as a therapeutic target

Altered brain CYP2B activity can functionally impact its CNS-acting substrates, previously demonstrated for propofol (Khokhar, 2011) and chlorpyrifos (Khokhar, 2012), and confirmed for nicotine in this body of work (Garcia et al., 2015). Therefore, altering brain CYP2B could have therapeutic value: inhibiting brain CYP2B activity could increase the brain levels of drugs to increase their action and or to reduce or prevent the formation of active toxic metabolite(s), while increasing brain CYP2B activity could reduce the brain levels of drugs that would have negative effects in the brain or increase the brain activation of pro-drugs into their active metabolite(s). This could lead to lower drug doses required to provide therapeutic effect and/or to prevent or reduce damage when exposed to toxins.

4.2.1 Brain CYP2B inhibitors

Many CYP2B inhibitors can cross the blood-brain barrier, including the anti-depressants sertraline, paroxetine, norfluoxetine and fluvoxamine (Walsky et al., 2006) and the MBI C8X (Khokhar, 2014), indicating that peripheral administration of inhibitors to inhibit brain CYP2B is possible. As central inhibition would reduce CYP2B metabolism and increase brain drug levels of the CYP2B substrate, hepatic inhibition by peripheral administration of inhibitor would not adversely affect brain drug levels because inhibiting hepatic metabolism would increase systemic levels of the drug that could then enter the brain. Higher brain levels of CYP2B substrates that act in the CNS with inhibition would mean lower doses of the drug could be used to elicit the drug's therapeutic effect, for example inhibiting CYP2B activity in the liver could increase propofol levels systemically at a given dose which could increase propofol levels entering the brain, possibly increasing its anaesthetic affect. Inhibiting CYP2B activity specifically in the brain would lead to increasing propofol levels that have entered the brain by reducing its local metabolism, which could also contribute to increasing propofol anaesthesia. As brain propofol levels are a better predictor of propofol's anaesthetic effect compared to plasma propofol levels (Liu et al., 2009), inhibiting CYP2B activity in the brain may be a more desirable method of increasing propofol anaesthesia.

In the case of CYP2B substrates that are converted into active toxic metabolites, such as chlorpyrifos, inhibiting hepatic metabolism would also reduce formation of toxic metabolite(s), suggesting that inhibitors could be used as a treatment for toxic exposure. Recent work from our group demonstrated that peripheral administration of C8X was able to inhibit both hepatic and brain CYP2B activity and reduce chlorpyrifos-mediated neurotoxicity (Khokhar, 2014). C8X was given intraperitoneally, which is similar to an oral route of administration in that the drug undergoes first-pass metabolism in the liver (Turner et al., 2011), suggesting that the inhibitor can be given peripherally and reach the brain in sufficient amounts to inhibit CYP2B.

Nasal delivery of drugs is one method by which the inhibitor can be administered directly to the brain. This method involves the uptake of drug intra-nasally through branches of the trigeminal nerve, the olfactory bulb, or through central blood circulation through capilliaries in the nasal cavity (Kozlovskaya et al., 2014). This would be useful to inhibit CYP2B activity in the brain and influence local CYP2B metabolism without altering systemic levels of the CYP2B substrate, especially if inhibitors cannot cross the blood-brain barrier. If we take the example described previously with the substrate propofol, nasal delivery of inhibitor would be expected to reduce CYP2B activity specifically in the brain and subsequently increase central propofol levels to prolong unconsciousness. This would then reduce the amount of drug required to maintain this effect.

4.2.2. Brain CYP2B inducers

Phenobarbital (Schilter et al., 2000; Lee et al., 2006b), phenytoin (Kempermann et al., 1994), and nicotine (Miksys et al., 2000a; Lee et al., 2006a) are some examples of brain CYP2B inducers. Phenobarbital (Botelho et al., 1979; Chang et al., 1997; Schoedel et al., 2003) and phenytoin (Diwan et al., 1988; Wang et al., 2004) can induce brain and liver CYP2B but nicotine induces brain but not liver CYP2B (Miksys et al., 2000a; Lee et al., 2006a). Peripheral administration of inducers, such as phenobarbital and phenytoin, also induce hepatic CYP2B, which could reduce the harmful effect of a drug in the brain by increasing liver metabolism but could also lead to reduction in the potential therapeutic effect of the drug. Thus, inducers given specifically into the brain could be used to reduce or prevent harmful effects of CYP2B-inactivated substrates without altering systemic levels of drug. One example of how brain CYP2B induction could reduce harmful drug effects would be the anti-HIV drug efavirenz. CNS side effects, which

include mood and sleep disturbances, are reported in 40-60% of individuals undergoing efavirenz treatment (Apostolova et al., 2015). CNS toxicity from efavirenz entering the brain is thought to be responsible for these side effects, with recent evidence suggesting that efavirenz inhibits mitochrondrial function in neurons leading to oxidative stress and disturbances in neuronal function (Funes et al., 2014). Therefore, CYP2B induction specifically in the brain, and not in the liver, might reduce efavirenz levels in the brain by increasing local efavirenz metabolism, which in turn might reduce CNS toxicity while still providing plasma levels sufficient to reduce viral load.

Hepatic induction would also increase the activation of pro-drugs into their metabolite(s), which would be beneficial if the metabolite can cross the blood-brain barrier to exert its effects. If the metabolite cannot cross the blood-brain barrier, or requires active transport resulting in a delay in entry to the brain, then brain-selective induction of CYP2B would be beneficial to ensure that sufficient levels of the active metabolite are produced within the brain. One example by which brain specific CYP2B induction could increase the activation of a pro-drug that acts in the brain would be the smoking cessation drug bupropion. Bupropion is biotransformed by CYP2B into a hydroxyl-metabolite, hydroxybupropion (Hesse, 2000). Plasma levels of hydroxybupropion correlate with abstinence (Zhu et al., 2012), suggesting that this metabolite is responsible for mediating its effects on smoking cessation. The mechanism by which hydroxybupropion mediates cessation is unclear; however, the metabolite can inhibit nAChRs and the reuptake of dopamine and norepinephrine neurotransmitters (Damaj et al., 2004), suggesting it may reduce nicotine's effects on receptor activity. Although inducing CYP2B activity in the liver might increase hydroxybupropion formation systemically, which could increase its efficacy for smoking cessation, inducing CYP2B activity in the brain might increase hydroxybupropion formation locally for the metabolite to exert its actions within the brain. This change in local levels within the brain might then be useful for improving efficacy without changing the administered dose of bupropion.

Nicotine would be one inducer that could be given systemically because, in rats and monkeys, it increases brain CYP2B but does not alter hepatic CYP2B levels (Miksys et al., 2000a; Lee et al., 2006a). Higher CYP2B6 protein levels have been reported in post-mortem brains of smokers (Miksys, 2003), which suggests that nicotine-mediated induction in the brain might be possible in humans. Although it is a drug of abuse, its reinforcing effect is thought to be mediated by

rapid delivery to the brain by cigarette smoking or IV nicotine (Hukkanen et al., 2005). Therefore, if CYP2B induction in the brain can occur after chronic nicotine exposure, chronic nicotine given in a slow delivery where it is not reinforcing, such as the nicotine patch (Hukkanen et al., 2005), could be used to induce CYP2B. Intranasal delivery, described in the previous section, could also be used to induce CYP2B selectively in the brain by direct delivery of inducer into the CNS. This method might be useful to deliver CYP2B inducers that increase liver and brain CYP2B, such as phenobarbital, to specifically increase CYP2B activity in the brain leaving hepatic CYP2B unchanged.

4.2.3 Drugs that affect CYP2B gene regulation

4.2.3.1 Reducing gene expression

Pharmacological inhibitors of CYP2B typically inhibit enzyme activity by interacting with the protein; the majority of CYP2B inhibitors are MBIs (Walsky et al., 2006). There are, however, drugs that can inhibit expression of the CYP2B gene, which would be another method to reduce CYP2B activity. LY2090314, a glycogen synthase kinase-3 inhibitor, was able to reduce CYP2B6 catalytic activity in human hepatocytes by inhibiting CAR and reducing mRNA levels (Zamek-Gliszczynski et al., 2014). Inhibitor treatment did not alter the expression of other CYPs, such as CYP1A2 or CYP3A, at doses that reduced CYP2B6 mRNA, suggesting that its effect was selective for CYP2B6 and these same doses were not cytotoxic (Zamek-Gliszczynski et al., 2014), suggesting that the inhibitor can suppress CYP2B6 transcription without significantly affecting the cells. The drug metformin, used to treat diabetes, can also prevent CAR-mediated induction of CYP2B6 expression in human hepatocytes by enhancing CAR phosphorylation, which prevents it from translocating to the nucleus (Yang et al., 2014). This was shown by drugs that directly (CITGO [6-(4-chlorophenyl)-imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime]) and indirectly (phenobarbital) activate CAR (Moore et al., 2000). Therefore, pharmacological inhibition of both constitutive and induced CYP2B gene expression is possible, which could be utilized to reduce subsequent protein levels and activity of the enzyme.

4.2.3.2 Inducing gene expression

CYP2B inducers typically increase enzyme activity by increasing gene transcription (Wang and Negishi, 2003). CYP2B gene transcription can be induced by activation of the nuclear receptors CAR by phenobarbital or phenytoin (Chang et al., 1997; Wang et al., 2004), and PXR by rifampicin (Goodwin et al., 2001), or by chronic nicotine exposure, through mechanisms not yet characterized (Miksys et al., 2000a; Khokhar, 2010). Chronic nicotine induces CYP2B expression in the brain but not the liver in rats (Miksys et al., 2000a; Khokhar, 2010) and monkeys (Lee et al., 2006a) and higher CYP2B protein levels have been reported in the post-mortem brain of smokers (Miksys, 2003); therefore the mechanisms involved in this tissue-specific induction could lead to new targets to increase CYP2B activity in the brain.

Chronic nicotine treatment in monkeys utilized SC nicotine injections given twice daily for 22 days to achieve average daily amounts of nicotine similar to that in smokers (Lee et al., 2006a; Ferguson et al., 2013), which resulted in increased CYP2B protein in several brain regions but not in the liver. Increases in CYP2B mRNA were not detectable in monkeys, suggesting that non-transcriptional mechanisms, such as protein stabilization and/or reduction in protein degradation, could be involved (Ferguson et al., 2013). The chronic nicotine treatment paradigm in rats that induced brain CYP2B utilized SC nicotine injections given once a day for seven days, which increased CYP2B mRNA and protein in the brain (Miksys et al., 2000a). Increases in mRNA levels suggests that CYP2B induction could be due to an increase in mRNA transcription, an increase in the stability or half-life of mRNA, or a decrease in mRNA degradation, leading to more translated protein. This induction was independent of nAChR activation, as treatment with central nAChR antagonist chlorisondamine did not inhibit CYP2B induction (Khokhar, 2010). Although the mechanisms of CYP2B induction appear different between the two species, suggesting that the mechanisms of CYP2B induction in humans might also be different, the knowledge that brain CYP2B induction in the rat might be due to changes at gene transcription or mRNA translation provides a model to determine the molecular basis of these changes. These pathways can then be investigated to determine if they can be manipulated pharmacologically to induce brain CYP2B.

Nicotine mediates its pharmacological actions primarily through acting on nAChRs in the brain (Sargent, 1993). As chlorisondamine did not block CYP2B induction, it suggests that nicotine

induced CYP2B through mechanisms independent of receptor activation. Nicotine can mediate effects independent from receptor activation; one example would be its neuro-protective effect in neurotoxicity caused by the 1-methyl-4-phenylpyridinium ion (MPP+) (Xie et al., 2005). MPP+ is an active product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which enters dopamine neurons through the dopamine transporter and disrupts mitochondrial function by interfering with oxidative phosphorylation (Singer et al., 1988). Nicotine treatment of isolated mitochondria from rat brain prevented the MPP+-mediated disruption in mitochondria function, indicating that nicotine was protective (Xie et al., 2005). The effect of nicotine in preserving respiratory chain function was independent of nAChR activation as mecamylamine did not block this effect (Xie et al., 2005). This work suggests that nicotine might affect the activity of intracellular molecules through non-nAChR as well as nAChR mechanisms. Therefore, nicotine might modulate intracellular molecules that contribute to gene regulation.

The brain-specific induction in rat CYP2B by nicotine suggests that the gene is likely regulated differently in this tissue compared to the liver. The nuclear receptors CAR and PXR are expressed in the human (Lamba et al., 2004a; Lamba et al., 2004b) and rat (Bauer et al., 2004; Wang et al., 2010) brain, which suggests that inducers known to increase hepatic CYP2B levels through these nuclear receptors, such as phenobarbital or phenytoin, may also activate these nuclear receptors in the brain resulting in brain CYP2B induction. This implies that if nicotine activates these same transcriptional factors it may be through different molecular pathways than phenobarbital or phenytoin. The mechanism underlying phenobarbital induction of CYP2B in the liver is not fully characterized; however, it is believed to occur through indirect activation of CAR in rodents and humans (Honkakoski et al., 1998; Sueyoshi et al., 1999; Yoshinari et al., 2001). Phenytoin-mediated induction of CYP2B in the liver is also thought to be mediated through CAR activation in humans (Wang et al., 2004) and mice (Jackson et al., 2004). Thus, nicotine might activate brain-specific factors that could activate CAR and induce CYP2B expression. Alternatively, nicotine might activate brain-specific factors that could induce CYP2B expression independent of CAR.

CAR mRNA has been detected in the caudate nucleus of human brain (Lamba et al., 2004a) and in capillary endothelial cells of the blood-brain barrier in both human (Dauchy et al., 2008; Dauchy et al., 2009) and rat (Wang et al., 2010) brain. Phenobarbital can induce expression of drug transporters regulated by CAR in rat endothelial cells (Wang et al., 2010), suggesting that

CAR is functional at the blood-brain barrier. Nicotine would not be expected to directly activate CAR because CYP2B is not induced by nicotine in the rat liver. CYP2B6 expression can be induced by cigarette smoke extract in cultured human hepatocytes expressing CAR (Washio et al., 2011), suggesting that nicotine might induce CYP2B6 through CAR in humans, but other constituents in smoke extract can activate CAR, such as the aryl-hydrocarbon receptor ligand benzo[a]pyrene (Patel et al., 2007). Therefore, if nicotine induces CYP2B in the brain through CAR activation, this is likely due to nicotine activating brain-specific factors that can then interact with CAR. CAR activation in the rat liver may be mediated in part by adenosine monophosphate (AMP)-activated protein kinase alpha (AMPK), as AMPK activators can mediate CAR translocation in the rat liver, although they do not increase CYP2B induction (Shindo et al., 2007). Nicotine increases AMPK activation in mouse adipocytes (An et al., 2007), but inhibits AMPK in the rat hypothalamus (Martinez de Morentin et al., 2012), indicating that nicotine has different effects on intracellular pathways in different tissues. This suggests that if CAR activation is involved in CYP2B induction in the brain, then nicotine may indirectly activate CAR through a different pathway than AMPK activation compared to the liver, where AMPK activation activates CAR.

Nicotine might indirectly activate CAR in the brain through an intracellular cyclic AMP (cAMP) signaling pathway. In the liver, cAMP can modulate the inducibility of CYP2B: cAMP analogs can inhibit CYP2B induction by phenobarbital in rat hepatocytes (Sidhu and Omiecinski, 1995). This would be consistent with the role of AMPK in CAR activation as cAMP signaling through protein kinase A (PKA) activity can inhibit AMPK activation in the liver (Aw et al., 2014). Given that nicotine has opposite effects on AMPK activation in the liver versus the brain, it is possible that cAMP signaling in the brain is also different from the liver. Chronic nicotine can down-regulate phosphodiesterase 4 (PDE4), which regulates cAMP (Bender and Beavo, 2006), in the rat brain in a region-specific manner (Polesskaya et al., 2007). Consistent with this, post-mortem brains from human smokers show elevations in cAMP signaling proteins: greater protein kinase a (PKA) protein in the VTA and greater PKA and adenylyl cyclase activity in nucleus accumbens and VTA tissue (Hope et al., 2007). Therefore, if different downstream effects of cAMP signaling can occur in the brain versus the liver it is possible that CAR could be regulated by nicotine in the brain by these calcium dependent intracellular pathways. Various isoforms of two downstream components in cAMP signaling, PKA and A kinase anchoring proteins

(AKAPs), display differences in their tissue and brain-region expression (Taskén and Aandahl, 2004) , which could contribute to different effects in cAMP-mediated signaling. This would be consistent with the tissue specificity of nicotine induction, where cAMP signaling may prevent induction in the liver but facilitate induction in the brain. As PDEs, particularly the PDE4 isoforms, can regulate cAMP (Bender and Beavo, 2006), it would be interesting to examine the effect of inhibiting these enzymes on CYP2B expression. PDEs have diverse expression throughout the human (Lakics et al., 2010) and rat brain (Iona et al., 1998; Polesskaya et al., 2007), where different forms are expressed in a region-specific manner, which suggests that cAMP regulation could be different within different regions of the brain. PDE4 inhibitors are available and currently prescribed for a number of inflammatory respiratory diseases (Spina, 2008), which would provide a way to selectively inhibit this PDE in the brain by ICV injection.

CYP2B can also be induced by PXR activation in the human liver (Goodwin et al., 2001). PXR mRNA has been reported in the thalamus, pons, medulla, and spinal cord in humans (Lamba et al., 2004b) and in capillary endothelial cells of the blood-brain barrier in rodents (Bauer et al., 2004). Human splice variants exist for PXR in the brain (Lamba et al., 2004b), suggesting that PXR expression and activity could vary within the brain and between the brain and liver. This would be consistent with the difference in PXR activation by rifampicin between these two tissues: rifampicin can activate PXR and induce CYP2B expression in human (Chang et al., 1997) and pig (Nannelli et al., 2010) liver; however, it does not induce CYP2B in pig brain (Nannelli et al., 2010). PXR can bind to the PBREM in mouse CYP2B10 using reporter plasmids expressed in cultured primate cells (Xie et al., 2000), suggesting that PXR can induce rodent CYP2B; however, one study using reporter plasmids with rat PXR suggests that it does not play a major role in liver CYP2B induction in this species (Cui et al., 2005). Thus, if PXR is involved in CYP2B induction in the brain by nicotine it could explain why CYP2B induction by nicotine does not occur in the liver. Brain PXR may have a functional role in the rat through the production of neurosteroids, such as 5α-pregnan-3α-ol-20-one (3α, 5α-THP) from cholesterol. PXR knockdown in the VTA reduced 3α, 5α-THP levels in the rat brain and resulted in lower anxiety and reproductive behaviour (Frye et al., 2013), which is consistent with 3α, 5α-THP's role in affect and motivation in reproductive behaviour (Frye and Rhodes, 2006). This suggests that PXR may be involved in the formation of this neurosteroid, demonstrating that it could play a role in the brain. Nicotine can activate expressed human PXR in a mouse fibroblast cell line

(Lamba et al., 2004b), suggesting that PXR activation could be a possible mechanism of CYP2B induction. Nicotine activation of rat PXR would first need to be tested, which could be carried out using expressed rat PXR in cell culture. To test whether PXR is involved in brain CYP2B induction *in vivo*, rats could be treated with PXR knockdown using antisense oligonucleotides previously characterized in rat brain by Frye et al. (2013). CYP2B induction was observed in the frontal cortex and brain stem after 7-day nicotine treatment (Miksys et al., 2000a; Khokhar, 2010), therefore antisense oligonucleotides could be microinjected into these two regions. Rats could be treated with the antisense oligonucleotides before 7-day nicotine treatment and then CYP2B protein levels could be measured after nicotine treatment to determine whether induction was impaired.

The retinoid X receptor (RXR) binds to PXR to form a heterodimer that binds to the PBREM on the CYP2B gene (Goodwin et al., 2001). RXRα mRNA has been detected in human brain (Kojo et al., 2004), which suggests that, if functional, it could interact with other transcription factors, such as PXR, in the brain and induce CYP2B expression. RXR is capable of binding to other nuclear receptors other than PXR, such as peroxisome proliferator-activated receptor α (PPARα) (Kliewer et al., 1992) which is expressed in the brain (Feng et al., 2008); however ligands that activate PPARα can induce CYP2B1/2 in rat liver (Shaban et al., 2005). The PPARα ligands oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) can reduce NSA and nicotine reinstatement behaviour in rats (Mascia et al., 2011), which would be consistent with induced CYP2B activity in the liver reducing nicotine plasma levels leading to a reduction in nicotine reinforcement. RXRα also interacts with nuclear receptor 1 protein (NURR1) (Perlmann and Jansson, 1995), a nuclear receptor that plays an important role in the development of dopamine neurons (Sacchetti et al., 2006). Chronic nicotine treatment increases NURR1 mRNA levels in the rat dorsal striatum (Saint-Preux et al., 2013), suggesting that this receptor could be another possible contributor to CYP2B induction in the brain. NURR1 was originally found to bind to the nerve growth factor IB (NGFI-B) response element (NBRE) as a monomer (Wilson et al., 1991; Murphy et al., 1996) and has subsequently been shown to bind as a heterodimer with RXR to a DR5 sequence motif related to NBRE (Perlmann and Jansson, 1995), as a dimer to a palindromic sequence labeled as a Nur response element (NurRE) (Maira et al., 1999), and on multiple NBRE-like motifs located within the tyrosine hydroxylase (TH) promoter region (Kim et al., 2003). The NBRE motif (Wilson et al., 1992) can be found in the NR1 element of the

PBREM of CYP2B1(Pustylnyak et al., 2007), suggesting that NURR1 might induce CYP2B in the rat brain through binding to this element.

Nicotine might also induce CYP2B in the brain by stabilizing mRNA. Nicotine can induce expression of macrophage inflammatory protein-1 alpha, a chemokine involved in pulmonary inflammation in rat alveolar macrophage cells (Chong et al., 2002). The rate of mRNA transcription and mRNA half-life increased with nicotine treatment, indicating both transcriptional and post-transcriptional mechanisms were responsible (Chong et al., 2002). Therefore, CYP2B induction could be mediated by increased gene transcription by activating nuclear receptors in the brain or by increasing the half-life of CYP2B mRNA.

Together, these pathways could be manipulated to test whether they modulate CYP2B induction. This would provide new potential targets to increase CYP2B levels in the brain as well as targets that could decrease CYP2B levels, as compounds that inhibit the induction could also be investigated or developed. For drugs in which changes to CYP2B activity in the brain could alter their efficacy, these compounds could prevent reductions in therapeutic effect or toxicity.

4.3 Clinical relevance of brain CYP2B activity

CYP2B participates in the metabolism of many clinically used CNS-acting drugs, whereby higher or lower enzyme activity in the brain could impact their therapeutic effect. CYP2B inhibitors or inducers could be used to alter brain drug levels to increase efficacy or decrease toxicity of CNS-acting substrates, depending on the role of CYP2B in the drug's metabolism. As many CYP2B inhibitors and inducers are also clinically used drugs (Walsky et al., 2006) or substances found in the human diet (Drocourt et al., 2002; Appiah-Opong et al., 2007), it is likely that CYP2B activity can vary in individuals. Consistent with this, studies using human hepatocytes report large variation in CYP2B activity in vitro (Code et al., 1997; Ekins et al., 1998; Gervot et al., 1999; Stresser and Kupfer, 1999). Therefore, if inhibitors or inducers can reach the brain, this variation in CYP2B activity would also occur in the brain. This would have important consequences for individuals with *CYP2B6* genetic variation and smokers. *CYP2B6* slow metabolizers would have lower brain CYP2B expression similar to that in the liver, suggesting that CYP2B activity towards its substrates could be reduced in the brain. Conversely, smokers, who might have higher CYP2B protein in the brain (Miksys, 2003), could then have increased activity towards CYP2B substrates. The impact of this variation on substrate action

would be dependent on whether the substrate is a therapeutic or a toxic compound and whether it is activated or inactivated by CYP2B.

4.3.1 Effect of CNS-acting CYP2B substrates

CYP2B6 slow metabolizers could have greater brain drug levels following administration of CYP2B substrates that enter the brain. Conversely, greater CYP2B levels are hypothesized in the brain of smokers, based on the observations from smoker post-mortem brain (Miksys, 2003), suggesting that smokers could have lower brain drug levels of CYP2B substrates that enter the brain. This could alter the therapeutic effect of CYP2B drug substrates that act in the CNS in smokers, as CYP2B activity could be higher in the brain and not the liver (Miksys, 2003). Thus, if brain-specific CYP2B activity is higher than normal it could possibly reduce the therapeutic effect of the drug by lowering its levels locally within the brain. Using propofol as one example, *CYP2B6* slow metabolizers could have higher brain propofol levels than expected from the peripheral dose administered, which would increase its anaesthetic effect. Thus, *CYP2B6* slow metabolizers could theoretically achieve anaesthesia at lower propofol doses. One recent study found individuals genotyped for the G516T mutation required lower propofol doses (Mourao et al., 2015), suggesting that *CYP2B6* slow metabolism in the liver increased plasma propofol levels sufficient enough to increase the anaesthetic response. The opposite was observed in a study with smokers, where they found higher doses were required to elicit anaesthesia (Lysakowski et al., 2006). This suggests that smokers, due to higher levels of CYP2B expressed in the brain, may metabolize propofol faster in the brain reducing propofol's anaesthetic effect. Thus, variation in CYP2B activity within the brain (in addition to the liver) may also influence propofol response.

CYP2B can participate in the metabolism of many CNS-acting drugs that are analgesics, anxiolytics, anti-depressants, anti-convulsants, and drugs of abuse. Thus, CYP2B variation in the brain could also influence the efficacy of these drugs similar to that seen with nicotine, in our experiments, and propofol in previous experiments (Khokhar, 2011). For *CYP2B6* slow metabolizers, reduced metabolism of these substrates in the liver and brain could increase the drug effect. Genetic polymorphisms in *CYP2B6* have been investigated with substrates by which CYP2B6 plays a large role in their metabolism, such as propofol (Court et al., 2001), efavirenz (Ward et al., 2003), and bupropion (Kirchheiner et al., 2003), where substrate plasma are

influenced by this variation. For substrates where CYP2B6 plays a minor role in the metabolism of its substrate in the liver, altered CYP2B6 activity could have a greater impact on metabolism in other tissues if it is expressed either in the absence of the major enzymes that metabolize the substrate or in higher amounts compared to these major enzymes. This may explain the impact of genetic *CYP2B6* slow metabolism in smoking, where CYP2B6 protein is expressed in the brain and potentially capable of nicotine metabolism, but only CYP2A6 mRNA has been detected in the brain (Miksys and Tyndale, 2004; Toselli et al., 2015b) at low levels below quantification (Toselli et al., 2015b). In this case, altered CYP2B6-mediated metabolism, locally in the brain, might alter the substrate's effects without altering systemic levels, which is largely mediated by hepatic expression of the major enzyme responsible for metabolizing the substrate. To my knowledge, other than the study that found *CYP2B6* genetic variation in smoking cessation (Lerman et al., 2002; Lee et al., 2007b), there have been no studies conducted to investigate whether *CYP2B6* genetic variation can influence drug response without influencing plasma drug levels.

Altered metabolism in the brain without influencing systemic levels could also occur when inhibitors or inducers influence brain CYP2B6 activity without influencing activity in the liver, like that seen with nicotine (Miksys et al., 2000a; Miksys, 2003; Lee, 2006). This suggests that smokers, who might have higher CYP2B6 levels in the brain, could have greater metabolism for CYP2B substrates. CYP2B can metabolize many anti-depressants (Turpeinen and Zanger, 2012), where greater CYP2B activity in the brain could reduce their effects if the parent drug is the active compound. Smoking is highly co-morbid with depression (Luger et al., 2014), which indicates that co-use of nicotine and anti-depressants may occur. Despite this, smoking status is not a common factor in clinical trials for anti-depressants (Weinberger et al., 2011), except for bupropion which is both an anti-depressant and smoking cessation agent. In a study that investigated the effect of combined treatment with mecamylamine and the SSRI sertraline in depressed individuals, the subjects that were smokers had less anti-depressant response compared to non-smokers (George et al., 2008). A lower anti-depressant response would be consistent with greater CYP2B activity in the brains of smokers from nicotine exposure, which would reduce available sertraline in the brain. The contribution of mecamylamine might confound this effect, as the nAChR system has been implicated in depression (Weinberger et al., 2011), which may contribute to the mechanisms underlying comorbidity between smoking and

depression. However, this reduction in anti-depressant response in smokers compared to non-smokers was also seen in the mecamylamine placebo group (sertraline with placebo), suggesting that smokers had a lower anti-depressant response to sertraline alone (George et al., 2008). This study was a preliminary investigation of the effect of combined nAChR antagonist and SSRI treatment (N=21), so the group of smokers and non-smokers in the placebo group are small (George et al., 2008), however it does suggest the possibility that smoking could influence drug response of other CYP2B substrates. Therefore, given that CYP2B6 is involved in the metabolism of nicotine and many anti-depressants, variation in enzyme activity might be an important factor in their efficacy within the brain.

4.3.2 Adverse effects of CYP2B substrates in the CNS

Altered CYP2B activity in the brain could have functional consequences for CYP2B drug substrates that may not primarily act in, but can, enter the brain. The anti-HIV drug efavirenz is another example of a CYP2B6 substrate that may be influenced by brain CYP2B variation. Efavirenz is a reverse transcriptase inhibitor used to prevent HIV replication and CYP2B6 is the main enzyme that inactivates efavirenz (Ward et al., 2003). *CYP2B6* slow metabolism has been associated with higher efavirenz plasma levels in multiple studies (Haas et al., 2004; Tsuchiya et al., 2004; Rotger et al., 2007; Gounden et al., 2010), indicating that CYP2B6 activity in the liver can influence efavirenz pharmacokinetics and subsequently its effect on reducing HIV viral load. Two of these studies also reported an association between *CYP2B6* slow metabolism and greater CNS side effects (Haas et al., 2004; Gounden et al., 2010), suggesting that greater brain efavirenz levels might contribute to the presentation of these side effects. CNS-mediated side effects reported in greater than 50% of patients who start treatment (Staszewski et al., 1999; Marzolini et al., 2001; Haas et al., 2004). These side effects usually last for a couple of weeks but can last for months (Lochet et al., 2003) and could be a reason for discontinuation or switching to another anti-HIV drug (Clifford et al., 2005). Haas et al. (2004) investigated the effect of *CYP2B6* genotype on CNS side effects throughout the time course of efavirenz treatment. This group found a significant association between *CYP2B6*6* (slow metabolism allele) and presentation of CNS side effects at week 1 of treatment, but not at later time points. Efavirenz can induce hepatic CYP2B6 activity *in vivo*: efavirenz treatment can increase bupropion metabolism in healthy controls (Robertson et al., 2008), suggesting that efavirenz

treatment may subsequently induce CYP2B6 activity in the liver and reduce plasma drug levels, thereby reducing side effects after continued treatment.

Given that these side effects occur early within treatment, reducing their appearance and/or duration could improve treatment adherence. Positive smoking status is associated with lower discontinuation (Wyen et al., 2011), which suggests that potentially higher brain CYP2B6 levels from smoking could reduce efavirenz levels locally in the brain and possibly diminish CNS side effects. Thus, while altered CYP2B activity in the liver and brain through genetic polymorphisms could influence efavirenz efficacy and CNS side effects, altered enzyme activity specifically in the brain could influence CNS side effects, which would be important for continuing treatment. If brain CYP2B is induced by nicotine, perhaps a co-treatment of nicotine patch and efavirenz during the early stages of treatment could reduce CNS side effects (via CNS inactivation of efavirenz) and reduce the burden and risk of discontinuation.

CYP2B6 can also bio-activate environmental compounds, such as chlorpyrifos and 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47), into toxic metabolites. Chlorpyrifos was shown to be activated into its toxic metabolite by previous work from our group (Khokhar, 2012), demonstrating that local activation by CYP2B6 in the brain could result in chlorpyrifos-mediated neurotoxicity. CYP2B6 in human hepatocytes can metabolize BDE-47 into hydroxylated BDEs that can disrupt Ca_{2+} signaling (Feo et al., 2013). These hydroxylated BDEs were shown to be neurotoxic using rat cortical neuronal cultures (Kim et al., 2011), modulating GABA and nAChR systems (Hendriks et al., 2010), suggesting that local metabolism of BDE-47 within the brain could activate these metabolites and alter brain function. Variation in brain CYP2B would either protect or increase the risk of toxicity by these environmental compounds, where *CYP2B6* slow metabolizers would have less toxic metabolite produced while individuals with induced CYP2B activity in the brain, such as smokers, would have increased levels of toxic metabolite. Therefore, altered CYP2B activity could modulate the risk for adverse effects of its substrates within the brain.

5 Summary

These experiments demonstrated (1) factors that influence acquisition, motivation, and nicotine-seeking behaviour in the rodent nicotine self-administration model, (2) a role for brain CYP2B activity in nicotine self-administration behaviour, and (3) a role for brain CYP2B activity in modulating CYP2B substrate levels, such as nicotine, in the brain. The experiments examining nicotine self-administration design provided the parameters by which the effect of inhibiting brain CYP2B activity could be tested and provided information on NSA acquisition not fully characterized in the literature. The effect of brain CYP2B inhibition on NSA acquisition and motivation was consistent with that seen in smokers who are *CYP2B6* slow metabolizers, supporting our hypothesis that variation in brain enzyme activity could contribute to differences in smoking behaviour. Finally, the effect of both inhibiting and inducing brain CYP2B activity on nicotine levels in the brain demonstrated that changes in brain CYP2B activity could significantly alter the circulating levels of its substrates. Together, this work suggests that brain CYP2B activity is clinically relevant and that variation in this local activity could be one factor contributing to inter-individual variation in drug response.

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