Brain CYP2D Metabolism of Opioids Impacts Brain Levels, Analgesia, and Tolerance

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Pharmacology and Toxicology
University of Toronto

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Doctor of Philosophy
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University of Toronto
2018

Abstract
Cytochrome P450 2D (CYP2D) is a subfamily of enzymes expressed in both liver and brain that metabolizes clinically used drugs, neurotoxins, and endogenous neurochemicals. Opioid analgesics are metabolized by CYP2D to more potent analgesic metabolites, and variation in this metabolism may alter opioid response. While human CYP2D expression in the liver is primarily regulated by genetics, brain CYP2D activity may be altered by genetics, environmental inducers, and hormonal regulation. Smokers have higher brain, but not liver, CYP2D and nicotine induces rat brain, but not liver, CYP2D. This thesis investigated the impact of altering rat brain CYP2D activity in vivo on the brain metabolism of opioids and resulting analgesia. Rats were administered opioids and analgesia was measured; drug levels were assessed in brain and plasma after sacrifice, or in brain in vivo by microdialysis. Rat brain CYP2D was inhibited by intracerebroventricular injection of a CYP2D mechanism-based inhibitor or induced by seven-day subcutaneous nicotine treatment. We found that rat brain CYP2D activation of codeine to morphine mediates acute codeine analgesia; inhibiting brain CYP2D decreased, and inducing brain CYP2D increased, brain morphine from codeine and resulting codeine analgesia. In a rat model of codeine analgesic tolerance, inducing brain CYP2D increased acute analgesia and the rate of analgesic tolerance, producing a greater absolute decrease in analgesia per codeine dose. Administering higher doses of codeine similarly increased acute analgesia and the rate of tolerance. The rate of tolerance correlated to acute analgesia indicating that brain CYP2D influenced codeine tolerance by altering acute analgesic-response. Lastly, we found that rat brain CYP2D metabolism of oxycodone to oxymorphone decreased acute oxycodone analgesia; inhibiting brain CYP2D increased brain oxycodone levels and resulting analgesia, while inducing brain CYP2D increased brain oxymorphone levels but decreased oxycodone analgesia. This thesis demonstrated an impact of rat
brain CYP2D on acute codeine and oxycodone induced analgesia, as well as the rate of codeine analgesic tolerance. Variation in human brain CYP2D metabolism may be a clinically relevant source of variation in opioid response. Novel approaches to understanding interindivudial differences in opioid analgesia and adverse effects remain critical for improving opioid efficacy and mitigating abuse.
Acknowledgements

First, I wanted to thank my supervisor Rachel Tyndale for her support throughout my research. Your mentorship has helped immeasurably in developing my skills in scientific writing, critical thinking, presenting, and independent research. The training and opportunities I have been given will be invaluable moving forward in my career and I am very grateful. I also wanted to thank my PhD supervisory committee members David Riddick and Jose Nobrega for sharing their scientific expertise by way of continued insight and helpful suggestions.

I also wanted to extend many thanks to all past and present members of the Tyndale lab. Specifically, thanks to Sharon Miksys for her kindness and approachability in all matters scientific and otherwise, Bin Zhao for his resilience in running my thousands of samples, Fariba Baghai Wadji for her masterful surgical hands, Ewa Hoffman for providing welcomed distractions and an outlet for my hockey addiction, Meghan Chenoweth for ensuring I was always well-caffeinated, and JAT for leading the way and providing me with a graduate school blueprint which without, I would be nowhere near finishing this PhD.

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Submitted Abstracts for Oral and Poster Presentations

McMillan, D.M., Tyndale, R.F. Poster: Variation in rat brain CYP2D alters oxycodone metabolism and analgesia. May 2017 – UofT Department of Pharmacology Visions in Pharmacology; Toronto, ON, Canada. October 2016 – Annual symposium on Microsomes and Drug Oxidations; Davis, CA, USA.


McMillan, D.M., Tyndale, R.F. Oral: Nicotine increases codeine analgesia through the induction of brain CYP2D and central activation of codeine to morphine. April 2015 – Campbell Family Research Institute, Center for Addiction and Mental Health, Trainee Seminar Series; Toronto, ON, Canada.

McMillan, D.M., Tyndale, R.F. Poster: Nicotine increases codeine analgesia through induction of brain CYP2D and central activation of codeine to morphine. March 2015 – Annual Meeting for The American Society for Pharmacology and Experimental Therapeutics (ASPET) in conjunction with Experimental Biology (EB); Boston, MA, USA.

McMillan, D.M., Zhao, B., Tyndale, R.F. Poster: Chronic nicotine increases codeine analgesia through induction of brain CYP2D metabolic activation of codeine to morphine. May 2014 – Department of Pharmacology Visions in Pharmacology (VIP); Toronto, ON, Canada.
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<th>Definition</th>
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<tbody>
<tr>
<td>%MPE</td>
<td>Percentage of maximum possible effect</td>
</tr>
<tr>
<td>8MOP</td>
<td>8-Methoxypsoralen</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DAMGO</td>
<td>(D-Ala², N-MePhe⁴, Gly-ol)-enkephalin</td>
</tr>
<tr>
<td>DOR</td>
<td>Delta (δ) opioid receptor</td>
</tr>
<tr>
<td>EM</td>
<td>Extensive metabolizer</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma (γ)-aminobutyric acid</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein-coupled receptor kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HNF4a</td>
<td>Hepatocyte nuclear factor 4-alpha (α)</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IM</td>
<td>Intermediate metabolizer</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>KOR</td>
<td>Kappa (κ) opioid receptor</td>
</tr>
<tr>
<td>LCMS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>MBI</td>
<td>Mechanism-based inhibitor</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multiple drug resistance protein 1</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>MOR</td>
<td>Mu (µ) opioid receptor</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>1-Methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>O-DSMT</td>
<td>O-Desmethyl tramadol</td>
</tr>
<tr>
<td>OPRM1</td>
<td>Opioid receptor mu (µ) 1</td>
</tr>
<tr>
<td>OR</td>
<td>Opioid receptor</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal gray</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-Glycoprotein</td>
</tr>
<tr>
<td>PM</td>
<td>Poor metabolizer</td>
</tr>
<tr>
<td>p.o.</td>
<td>Per os (oral gavage)</td>
</tr>
<tr>
<td>PO</td>
<td>Prescription opioid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>RVM</td>
<td>Rostral ventromedial medulla</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TFL</td>
<td>Tail-flick latency</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine 5’-diphospho-glucuronosyltransferase</td>
</tr>
<tr>
<td>UM</td>
<td>Ultra-rapid metabolizer</td>
</tr>
<tr>
<td>VCM</td>
<td>Vacuous chewing movement</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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Appendix A: CYP-mediated drug metabolism in the brain impacts drug response…………185
Statement of Research Problem

Centrally-acting drug response does not always correlate with circulating drug or metabolite levels (Michels and Marzuk, 1993). This is due, in part, to permeability of the drug and/or active drug metabolites across the blood-brain barrier (BBB) and into the brain, but may also be due to drug metabolism within the brain affecting central nervous system (CNS) drug and/or metabolite levels. Drug-metabolizing cytochrome P450 (CYP) enzymes are expressed and active in the brain (Miksys and Tyndale, 2013); region- and cell-specific brain CYP expression and activity have been characterized in numerous experimental animals including rats (Miksys and Tyndale, 2002). The CYP2 family, including CYP2D, is expressed in both liver and brain, and metabolizes roughly 20% of all clinically used drugs, including a large proportion of CNS-acting drugs (e.g. antidepressants, antipsychotics), drugs of abuse (e.g. psychostimulants), and neurotransmitters (Bromek et al, 2010; Haduch et al, 2013; Zanger et al, 2004; Zanger and Schwab, 2013).

While hepatic CYP2D levels are primarily regulated by genetics, brain CYP2D levels are influenced by genetics, age, sex, and xenobiotic inducers (Mann et al, 2012; Miksys and Tyndale, 2013). Higher brain (but not liver) CYP2D is found in smokers, and seven days of nicotine administration induces rat brain (but not liver) CYP2D (Mann et al, 2008; Miksys et al, 2002; Yue et al, 2008; Zanger and Schwab, 2013). Variation (genetic and/or environmental) in brain CYP2D activity may contribute to clinically relevant differences in individual response to centrally acting CYP2D substrates that are not reflected in peripheral, hepatic CYP-influenced, drug and metabolite levels.

Opioid analgesics are an effective class of analgesic compounds, used widely in the treatment of pain associated with numerous indications (e.g. diabetic neuropathy [neuropathic], osteoarthritis [visceral], and myocardial ischaemia [somatic]) (Smith, 2011). Due to their rewarding and reinforcing properties, extended opioid treatment can lead to misuse, dependence, and mortality. The overall prevalence of opioid abuse and misuse among adult opioid users in the United States was 12.5% in 2016, relatively stable from 12.8% in 2015 (NSDUH, 2016, 2017). However, abuse prevalence ranges by opioid compound, from 10.4% of adult codeine users reporting abuse, 14.1% of oxycodone users, and 27.6% of oxymorphone users (NSDUH, 2017).

Interindividual differences in opioid pain relief and in risk for dependence are not fully understood. Most opioids, like codeine and oxycodone, are metabolically activated by CYP2D to
compounds that are more potent opioid receptor ligands, and thereby elicitors of analgesia. CYP2D metabolizes codeine to morphine, a step required for codeine analgesia; CYP2D poor metabolizers and individuals given CYP2D inhibitors produce little morphine from codeine and exhibit little codeine analgesia (Chen et al., 1991b; Sindrup et al., 1992). Oxycodone is also metabolized to the more potent analgesic compound oxymorphone; however, while giving individuals CYP2D inhibitors decreased the production of oxymorphone from oxycodone, oxycodone analgesia was unaffected (Gronlund et al., 2010). Investigation into opioid metabolism and analgesia in CYP2D poor metabolizers, as well as individuals given peripheral CYP2D enzyme inhibitors, indicate that depending on the opioid, CYP2D metabolism may promote, mitigate, or have no effect on, opioid response (Smith, 2011). Little is known about the role of brain CYP2D metabolism in opioid response.

Brain CYP metabolism may play a role in neuroprotection through metabolic inactivation of neurotoxins. Brain CYP2D inactivates toxins that can cause parkinsonism-symptoms such as pesticides; genetically rapid CYP2D6 metabolizers are at a lower risk for Parkinson disease, potentially due to enhanced brain CYP2D6-mediated inactivation of parkinsonism-inducing toxins (Elbaz et al., 2004; Zanger et al., 2004). Smokers also have a lower risk of Parkinson’s disease which may be due in part to greater CYP2D6 expression in the brain (Alves et al., 2004). In support of this, nicotine protects against the parkinsonism-inducing compound 1-methyl-4-phenylpyridinium (MPP+)-induced nigrostriatal damage in an animal model of Parkinson’s disease (Quik et al., 2009). Also, inhibiting CYP2D exacerbates MPP+ (neurotoxin)-induced cell death in a human neuronal cell line, further suggesting a role for brain CYP2D in protection against neurotoxicity (Mann and Tyndale, 2010). Manipulating brain, but not liver, CYP2D metabolism of haloperidol in rats alters neurotoxicity in both acute and chronic haloperidol administration paradigms (Miksys et al., 2017).

The studies included in the thesis aim to provide insight into the impact of brain CYP2D metabolism of opioids on the functional outcome of analgesia through altering brain CYP2D metabolism during acute or repeated opioid administration. This line of research stems from the finding that selectively inhibiting rat brain, but not liver, CYP2D decreased brain morphine levels and analgesia after acute subcutaneous codeine administration (Zhou et al., 2013). This thesis aims to extend these findings to models of variable (increased and decreased) brain CYP2D activity, routes of opioid administration more closely mirroring human use, multiple opioid compounds for
assessing opioid-specific influence of brain CYP2D metabolism, and multiple functional outcomes of opioid response. Investigation into this relatively new field of CYP function in the brain may provide a mechanistic understanding of a novel source of variation in opioid response, specifically codeine and oxycodone, and may have clinical implications in long-term opioid-use, escalation, and transition to abuse.
Main Research Objectives

Objective 1: CYP2D activates codeine to morphine, a step required for codeine analgesia. Differences in CNS permeability and efflux suggest that initial morphine in the brain after codeine dosing may be derived from brain CYP2D metabolism. While CYP2D in the liver is primarily regulated by genetics, a combination of genetics, environmental inducers, and age, may contribute to a wide range of brain CYP2D activity. Inhibiting rat brain CYP2D reduced brain morphine formation, and resulting analgesia, after subcutaneous codeine administration. However, it is unknown whether selectively inducing brain CYP2D changes brain CYP2D sufficiently to alter drug response in vivo, in the presence of substantial first-pass metabolism by the liver using intraperitoneal drug administration. Therefore, the first objective was to assess the effect of inducing (with and without concurrent inhibition) rat brain CYP2D activity, without altering liver CYP2D, on brain codeine and morphine levels, and analgesia, following intraperitoneal codeine administration.

Objective 2: Repeated exposure to opioids results in tolerance, a loss of analgesic potency, which may lead to dose escalation, a greater likelihood of opioid misuse, and transition to dependence. Inducing rat brain CYP2D increases the central activation of codeine to morphine, resulting in increased onset and magnitude of codeine analgesia (objective 1). It is unknown whether this difference in initial analgesia can affect the rate of development of tolerance. Higher initial codeine analgesia could result in an equivalent relative drop (equal slope of tolerance development), or a more rapid proportional drop (equal time to tolerance), in analgesic potency. Therefore, the second objective was to assess the effect of inducing brain CYP2D, and thereby the central activation of codeine to morphine, on the rate of development of tolerance to codeine’s analgesic effects across seven days of repeated oral codeine administration.

Objective 3: Oxycodone, similar to codeine, is metabolized by CYP2D to a more potent opioid receptor activating compound, oxymorphone. However, unlike codeine, oxycodone itself is a potent µ-opioid receptor agonist. Compared to its CYP2D metabolite oxymorphone, oxycodone has a greater influx across the BBB and is present at higher concentrations in the CNS after peripheral administration. While inhibiting human hepatic CYP2D does not alter oxycodone response, brain CYP2D metabolism may increase oxycodone analgesia through the formation of
oxymorphone directly within the CNS, may reduce oxycodone analgesia through local oxycodone metabolism, or may have no impact. The third and final objective was to assess the impact of rat brain CYP2D on the metabolism of oxycodone to oxymorphone, using in vivo brain microdialysis, and resulting analgesia after oral oxycodone administration.
1. General Introduction

1.1 – Opioid analgesics

1.1.1 General introduction

Opioid analgesics are among the most effective treatments available for pain. The term ‘opioid’ includes all compounds that bind to opioid receptors, while ‘opiate’ conventionally refers to opioid alkaloids derived from the opium poppy (*Papaver somniferum*) (Rosenblum *et al*., 2008). Opioids include semi-synthetic compounds derived from naturally occurring opiates (e.g. heroin from morphine). The history of human use of opioids dates back thousands of years, from records of exudation of opium from the opium poppy in the sixth millennium BCE, to the chemical isolation of the active alkaloid morphine from opium in the 19th century, and the clinical use of synthetic opioid analgesics today (Brook *et al*., 2017). While opioids are often regarded as the standard of care in treating acute and chronic pain, the associated side effects tolerance, and risks of abuse, addiction, and withdrawal have caused major concern about their long-term usefulness (Rosenblum *et al*., 2008). For this reason, the use of opioids for less severe indications, such as chronic non-malignant pain, remains controversial (discussed further in 1.1.5) (Manchikanti, 2008).

1.1.2 Current clinical use and treatment indications

Opioid analgesics are used to treat moderate to severe chronic pain, presenting in numerous disease states. For example, oxycodone has been indicated for neuropathic (e.g. diabetic neuropathy, post-herpetic neuralgia), somatic (e.g. osteoarthritis-related and rheumatic joint pain, low back pain), visceral (e.g. post-operative pain, myocardial ischaemia), and cancer pain (Riley *et al*., 2008). A meta-analysis of 41 randomized controlled trials up to 2006, found that opioids were effective in treating pain and improving quality of life in patients with nociceptive (benign, acute damage) and neuropathic (chronic nerve damage) pain (Furlan *et al*., 2006). While guidelines on formulations, dosing, and prescribing have been developed to better instruct opioid use in pain management, controversy still exists surrounding the type of conditions treated, and the safety in treating, with opioids (Dowell *et al*., 2016). This is due, in part, to the high prevalence of chronic pain in the general population; estimates vary, but up to 40% of Canadians (2011) and 30% of Americans (2010) reported experiencing some form of chronic pain in the previous year due to illness, an accident, or an existing medical condition (Johannes *et al*., 2010; Schopflocher *et al*., 2011).
Consequently, due to the high prevalence of chronic pain and the effectiveness of opioids in treating pain, opioids are prescribed regularly; in 2016, 92 million people above the age of 12 in the US reported using any prescription opioid, while US retail pharmacies dispensed approximately 250 million opioid prescriptions (NSDUH, 2017; Pezalla et al, 2017).

In Canada and the US, numerous opioid formulations have been commercialized for oral and transdermal administration, used primarily in ambulatory care settings, and for intravenous administration, used primarily in surgical settings. These include combination products of an anti-inflammatory (Acetaminophen, Acetylsalicylic acid (aspirin), Ibuprofen) with an opioid compound, such as codeine (Tylenol 1-4®), oxycodone (Percocet®, Percodan®), hydrocodone (Vicodin®, Vicoprofen®, Lorset®), or tramadol (Ultracet®) (Rosenblum et al, 2008). There are also single entity opioid formulations containing morphine (Avinza®, Kadian®, MS Contin®, MSIR®), codeine (Codeine Contin®), oxycodone (OxyContin®), fentanyl (Duragesic®, Actiq®, Fentora®), hydromorphone (Dilaudid®), oxymorphone (Opana®), and methadone (Dolophine®), to name a few (Rosenblum et al, 2008).

1.1.3 Pharmacology
1.1.3.1 Opioid receptors
There are three major families of opioid receptors (OR): µ-opioid receptors (MOR), δ-opioid receptors (DOR), and κ-opioid receptors (KOR). Beyond analgesia, opioid receptors are involved in other physiological functions, such as membrane ionic homeostasis, cell proliferation, emotional response, epileptic seizures, immune function, feeding and obesity, respiratory and cardiovascular control, and some neurodegenerative diseases (Feng et al, 2012). ORs are highly expressed in the CNS, though they are also found in many peripheral organs (e.g. heart, lungs, gastrointestinal tract) (Feng et al, 2012; Wittert et al, 1996).

All families of ORs are seven transmembrane G protein-coupled receptors (GPCRs) and show high sequence homology (>50%) between subtypes (Katritch et al, 2013). Upon ligand binding, receptor conformational changes result in intracellular binding of G\textsubscript{i/o} protein, guanosine triphosphate (GTP) replacement of guanosine diphosphate (GDP), and dissociation of the trimeric G\textsubscript{i/o} complex into G\textsubscript{α} and G\textsubscript{βγ} proteins (Stein, 2016). G\textsubscript{α} inhibits adenylyl cyclase and cyclic-
adenosine monophosphate (cAMP) production, whereas $G_{\beta\gamma}$ interacts directly with membrane ion channels (Stein, 2016). All three families of ORs modulate pre- and post-synaptic calcium ($Ca^{2+}$) channels, suppressing $Ca^{2+}$ influx, thereby decreasing neuronal excitability and reducing the release of pro-nociceptive neuropeptides (Wang et al., 2010). Furthermore, OR activation leads to the opening of G protein-coupled inwardly rectifying potassium and sodium ($Na^{+}$) channels, among others, summing to an overall decrease of neuronal excitation by neuron hyperpolarization, the prevention of action potential firing, and decreased transmission of stimulus (Luscher and Slesinger, 2010). Activation of each of the three OR subtypes produces analgesia, but with different side-effects, likely due to variable regional expression, plasticity, and activity of receptors in different parts of the central and peripheral systems (Stein, 2016). For example, respiratory depression and euphoria are associated with activation of MORs, convulsions and anxiolysis with DORs, and anti-inflammatory effects with KORs (Stein and Machelska, 2011; Zollner and Stein, 2007).

Endogenous opioid receptor ligands are derived from precursors proopiomelanocortin ($\beta$-endorphin), proenkephalin (met- and leu-enkephalin), and prodynorphin (dynorphins). In general, these peptides are not selective to one receptor subtype, due in part to 1) similarity in peptide structure, as most endogenous opioids contain an N-terminal tyrosine residue required for ligand-binding to ORs; 2) similarity in OR subtype structure and resultant intracellular signal transduction pathways; and 3) the formation of homo and heteromeric complexes between opioid receptors that modify their response to ligand binding (Ananthan, 2006; Feng et al., 2012; Law and Loh, 1999; Waldhoer et al., 2004). $\beta$-endorphin and the enkephalins act primarily at MORs and DORs to produce antinociception, while dynorphins can elicit both pro and antinociceptive effects by N-methyl-D-aspartate (NMDA) and KOR receptor activation, respectively (Stein, 2016). Endogenous opioids should be considered as adjuvants to opioid treatment, as preventing extracellular degradation of endogenous opioids by inhibiting peptidases (e.g. aminopeptidase N and neutral endopeptidase) in the CNS and periphery has been shown to produce analgesia in animal models (Roques et al., 2012).

The regional distribution of OR subtypes in the brain and in peripheral tissue have been documented across multiple species (including human and rat) using techniques including
immunohistochemistry, in situ hybridization, and radiolabeled ligand binding (Mansour et al, 1995; Peng et al, 2012). In general, these studies show high correlation between OR mRNA expression, protein expression, and ligand binding (Brodsky et al, 1995). Investigating region-specific mRNA levels in human post mortem brains, MOR was found to be widely distributed throughout the CNS with the highest transcript levels in the cerebellum; DOR transcript levels were greatest in the cerebral cortex, putamen, temporal lobe, and hippocampus; and KOR transcript levels were highest in the putamen (Peng et al, 2012). All three OR subtypes have high mRNA expression in the nucleus accumbens and caudate, consistent with their role in regulating pain perception (Peng et al, 2012). Differences in OR protein expression between species is also observed; for example, while all three OR subtypes have been identified and cloned in the rat brain, the rat cerebellum has abundant DOR but little-to-moderate MOR expression, while the human cerebellum has abundant MOR but little DOR protein (Mrkusich et al, 2004).

1.1.3.2 Opioid metabolism
Opioid compounds differ widely with respect to how they are metabolized, to what extent, and by which enzymes; however, there are several general patterns that can be discerned. Enzymatic metabolism of opioids produces more hydrophilic compounds (by dealkylation, hydrolysis, and oxidation) capable of undergoing renal excretion (Smith, 2009, 2011). Hepatic metabolism of opioids primarily involves structural modifications via cytochrome P450 (CYPs; phase I metabolism) and conjugation via uridine 5’-diphospho-glucuronosyl-transferase (UGT) enzymes (phase II metabolism) (Smith, 2011). These metabolites are often active and may interact to varying degrees with the same or different OR subtypes as the parent compound (Smith, 2011). Most opioids undergo first-pass metabolism in the liver, which may reduce or enhance their bioavailability (Somogyi et al, 2007). Phase I metabolism of opioids primarily involves CYP3A4 and CYP2D6 to different degrees depending on the opioid. For example, CYP3A4 is the primary metabolic enzyme for oxycodone in humans, producing noroxycodone by N-demethylation (45% of urinary recovery), while CYP2D6 facilitates O-demethylation of a smaller proportion of oxycodone to oxymorphone (11% of urinary recovery) (Klimas et al, 2013). CYP3A4 metabolizes more than 50% of all clinically used drugs, therefore drug-drug interactions can occur with co-use of CYP3A4 inducers and/or inhibitors and opioids, that are extensively metabolized by this enzyme (Smith, 2009). Individuals who ingested grapefruit juice, which contains bergamottin a
strong inhibitor of CYP3A4, for 5 days, had a 1.5-fold greater peak plasma oxycodone concentration and 1.7-fold greater overall oxycodone exposure (area under the concentration-time curve [0 – ∞]) after oral oxycodone administration (Nieminen et al, 2010). CYP2D6 also plays a prominent role in the metabolism of opioids, for example in the O-demethylation of codeine to morphine (15% by urinary recovery, compared to 10% metabolized through CYP3A4 to norcodeine) (Thorn et al, 2009). In general, CYP2D6 metabolically activates opioids to compounds with greater MOR affinity than the parent compound (e.g. morphine relative to codeine, and oxymorphone relative to oxycodone), whereas CYP3A4 metabolism of opioids produces compounds with less activity than the parent compound (e.g. norcodeine relative to codeine, and noroxycodone relative to oxycodone) (Somogyi et al, 2007). Consequently, research into the inter-individual variability in opioid response has primarily focused on variability in CYP2D6 activity (further discussed in 1.2, 1.3, and 1.4 inclusive).

Phase II metabolism of opioids represents another major metabolic pathway. Glucuronidation of opioids generally takes place on free hydroxyl groups, and preferentially on aromatic hydroxyl groups (e.g. C-3 of morphine) rather than alicyclic hydroxyl groups (e.g. C-6 of morphine) (Smith, 2011). Metabolism of morphine occurs primarily by UGT2B7 and UGT1A1 glucuronidation to morphine-3-glucuronide (60% of urinary recovery) and morphine-6-glucuronide (10% of urinary recovery) (Christolup, 1997); thus, genetic variation in UGT (e.g. UGT2B7) enzymatic glucuronidation of opioids may influence opioid excretion and response (Innocenti et al, 2008). Liver samples containing a UGT2B7 variant responsible for increased enzymatic activity showed increased morphine glucuronidation in vitro (45% versus samples without the variant) (Innocenti et al, 2008).

1.1.4 Opioid neuropharmacology
1.1.4.1 Analgesia
The neural pathway of pain starts at the dorsal horn of the spinal cord and ascends through the contralateral spinothalamic tract to the brain where pain processing occurs (Ossipov et al, 2010). Pain is processed as nociception, which refers to all thermal, mechanical, or chemical stimuli detected by nociceptors, whose cell bodies are located in the dorsal root ganglia (Basbaum et al, 2009). When noxious stimuli occur, nociceptors release glutamate, generating excitatory
postsynaptic currents in second order dorsal horns neurons; glutamate receptor activation stimulates action potential firing and transmission of the noxious stimulus to higher order neurons (Basbaum et al, 2009). Ascending projections target the thalamus, which sends projections to the lateral and basolateral amygdala, while collateral projections target the mesencephalic nuclei (rostral ventral medulla and periaqueductal grey) (Ossipov et al, 2010). Initial opioid analgesia can occur by direct activation of ORs expressed in the dorsal root ganglia, reducing pain transmission at this first synapse (Stein, 1995).

The primary mechanism behind opioid analgesia is descending modulation (supraspinal) with action at multiple brain regions that have high OR concentration, such as the periaqueductal grey (PAG), locus coeruleus, and rostral ventral medulla (RVM) (Ossipov et al, 2010; Pathan and Williams, 2012). The PAG-RVM-spinal cord pathway is an essential neural circuit for opioid-based antinociception or analgesia; the PAG receives input from the hypothalamus and amygdala, then synapses with the RVM, which in turn terminates in the dorsal horn (Figure 1) (Basbaum and Fields, 1984; Loyd and Murphy, 2009). Administration of morphine directly into the PAG produces MOR-dependent analgesia (reversible by naloxone, a MOR antagonist), as well as naloxone-reversible excitation of RVM neurons (Behbehani and Fields, 1979; Loyd and Murphy, 2009; Yaksh and Rudy, 1978). Similarly, lesioning the PAG, or administrating MOR antagonists, attenuates the antinociceptive effects of morphine administered systemically (Dostrovsky and Deakin, 1977; Loyd and Murphy, 2009; Ma and Han, 1991). Opioids are believed to increase descending inhibitory control (through the PAG-RVM-spinal cord circuit) of spinal nociceptive output by an indirect action (Kanjhan, 1995; Ossipov et al, 2010). Activation of ORs in the RVM involves excitation (or disinhibition) of off-cells, neurons that decrease action potential firing, and direct inhibition of on-cells, neurons that increase action potential firing (Kanjhan, 1995; Ossipov et al, 2010). Disinhibition of off-cells may involve direct OR-mediated presynaptic inhibition of GABAergic input, while on-cells are directly hyperpolarized by the electrophysiological consequences of OR activation (discussed previously in 1.1.3.1) (Li et al, 2015b).
Figure 1: Neural pathway of nociception and descending modulation by opioids. Ascending pain pathways are activated by noxious stimuli (red arrows). Opioid analgesics (blue circles) act on opioid receptors located in descending pain modulatory pathways (blue arrows) in the periaqueductal gray (PAG), rostroventral medulla (RVM), dorsal horn, and dorsal root ganglion (DRG) to downregulate ascending nociception (red arrows). Adapted from Ossipov et al. 2010.

Opioids may also act in the anterior cingulate cortex and insula, which are two components of the central limbic (emotional) system involved in encoding the emotional and motivational aspects of pain (Bushnell et al, 2013). Imaging studies show a relationship between emotional aspects of pain perception and neural activation of the anterior cingulate cortex and insula, as well as with endogenous activation of MORs in these regions, while lesioning these regions results in altered emotional responses to pain (Bushnell et al, 2013; Starr et al, 2009; Zubieta et al, 2001).

1.1.4.2 Tolerance
Repeated opioid administration results in tolerance, a reduction in opioid effect or drug potency, indicated by a rightward shift in the opioid dose response curve (Cahill et al, 2016); the rate of tolerance varies by opioid compound and by opioid pharmacological effect. For example, altered gut motility (constipation) exhibits minimal tolerance over time compared to other side-effects of repeated opioid use such as respiratory-depression, sedation, and nausea (Cahill et al, 2016). While
tolerance to opioids per se has not been demonstrated to be a reliable predictor of abuse liability, it presents a challenge for opioid treatment of chronic pain and relapse in opioid-dependent individuals (Cahill et al, 2016). Decreased analgesic potency and inadequate pain relief is a predictor of dose-escalation in pain patients. Tolerance to opioid-induced respiratory depression at higher opioid doses may cause mortality in opioid-dependent individuals who relapse after prolonged abstinence, due to loss of tolerance to opioid-induced respiratory side effects (Cahill et al, 2016; Siegel et al, 1982).

Numerous mechanisms contribute to opioid tolerance at a neurobiological level, including opioid receptor desensitization, phosphorylation, internalization, and down-regulation and the initiation of compensatory processes (like adenylyl cyclase superactivation) (Williams et al, 2013). OR phosphorylation is mediated by protein kinases (e.g. GPCR kinases, protein kinase C) and is a key initiating event for OR desensitization, i.e. the progressive loss of OR function after repeated exposure to an opioid (Bailey et al, 2009). Phosphorylation induces an OR conformation change that increases the affinity for β-arrestin proteins. Binding of β-arrestin to ORs uncouples G-protein signalling and recruits endocytotic machinery, leading to receptor internalization and degradation (Ferguson et al, 1996). Downregulation is a long-term cellular adaptation to repeated opioid administration, which may result from repeated degradation of internalized receptors and/or from a decrease in receptor synthesis (Koch and Hollt, 2008). Compensatory mechanisms that counteract OR function further contribute to opioid tolerance; for example, counterregulatory increases in adenylyl cyclase activity (adenyl cyclase superactivation) are seen after OR withdrawal and are thought to contribute to the development of opioid tolerance and subsequently to dependence (Chakrabarti et al, 1998a; Chakrabarti et al, 1998b).

Neural outcomes of prolonged opioid use (e.g. OR phosphorylation and desensitization) contribute to tolerance in a complex way and are agonist-dependent. For example, protein kinase C is involved in MOR phosphorylation (leading to desensitization) during morphine- but not DAMGO (MOR agonist)-mediated receptor activation, while MOR endocytosis mediated by β-arrestin occurs during DAMGO, but not morphine, activation (Celver et al, 2004; Johnson et al, 2006; Koch and Hollt, 2008). Agonists that do not induce endocytosis (like morphine) tend to
preferentially activate adaptive processes (e.g. adenylyl cyclase superactivation), leading to tolerance and subsequent dependence (Koch and Hollt, 2008).

1.1.4.3 Dependence
The biological mechanisms underlying opioid dependence involve OR activation (primarily MOR) in brain regions associated with reward and reinforcement, including the ventral tegmental area (VTA) (Le Merrer et al, 2009; Ting-A-Kee and van der Kooy, 2012). The VTA consists primarily of dopamine-producing cell bodies that project to the nucleus accumbens and further to the cortex, which mediate natural and drug-motivated behaviours; dopamine is the primary neurotransmitter implicated in positive reinforcement (Wise and Rompre, 1989). Rats and mice readily self-administer MOR-agonists into the VTA, and conditioned place preference produced by MOR agonists given systemically in rats is blocked by intra-VTA MOR-antagonist administration (Bozarth and Wise, 1981; Britt and Wise, 1983; Fields and Margolis, 2015). While VTA dopamine release has been regarded as the predominant theory of opioid-seeking motivation, dopamine-independent reinforcement may occur at the brainstem tegmental pedunculopontine nucleus (Ting-A-Kee and van der Kooy, 2012). However, relatively less is known about this non-dopaminergic VTA circuitry and its comparative impact is unclear (Ting-A-Kee and van der Kooy, 2012).

Regulation of VTA dopamine release is complex and multifactorial, involving dopaminergic, γ-aminobutyric acid (GABA)-ergic, and glutamatergic regulation (Figure 2). OR activation produces inhibition, either directly through hyperpolarization of neurons via activation of G protein-coupled inwardly rectifying potassium and Na+ channels, or indirectly through inhibiting neurotransmitter release (Williams et al, 2001). The majority of MORs in the VTA are expressed on GABAergic cells (Fields and Margolis, 2015). MOR agonists promote the release of dopamine in the VTA via disinhibition, i.e. removal of tonic GABAergic inhibition of dopamine neurons (Fields and Margolis, 2015; Kelley et al, 1980). This ‘two neuron’ model of opioid reward has been assessed in vitro; in rat VTA tissue, half of the immunocytochemically identified GABA neurons were inhibited (hyperpolarized) by DAMGO (Margolis et al, 2012). However, MOR activation in the VTA also inhibits GABA release onto non-dopaminergic neurons (Xia et al, 2011), and inhibits pre-synaptic glutamate release from terminals synapsing onto VTA neurons (Margolis et al, 2005). Despite this, glutamate signaling appears to be required in opioid reward; disrupting glutamate
neurotransmission in rat VTA blocked morphine-induced VTA dopamine release, and the acquisition of morphine CPP (Harris et al, 2004). Therefore, all physiological input contributing to opioid-induced VTA dopaminergic transmission and ultimately opioid reward and reinforcement is yet to be fully understood.

Figure 2: Neural pathway of opioid dependence. Opioid analgesics (blue circle) increase dopamine transmission (red arrows) from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), and further the prefrontal cortex, by disinhibition (blue arrow) of GABA cells acting on dopaminergic projections from the VTA. Adapted from Ting-A-Kee and van der Kooy 2012.

1.1.5 Opioid epidemic; misuse and abuse

Non-medicinal prescription opioid (PO) use and abuse in Canada and the United States has increased rapidly over the past three decades, along with the associated personal, economic, and healthcare burden from opioid morbidity and mortality, leading to what has been termed the “opioid epidemic” (Florence et al, 2016; Han et al, 2015). In 2016, more than 11 million people in the United States (12.5 % of PO users) reported using POs non-medicinally, defined as use of a medicine for a purpose other than as directed or indicated, whether willfully or unintentionally (NSDUH, 2017; Tetrault and Butner, 2015). Similarly, in Canada, POs are the second most prevalent drugs used illicitly (Fischer and Rehm, 2017). In 2014, US retail pharmacies dispensed 245 million opioid prescriptions, with between 9 and 11 million people receiving longer-term opioid therapy (Volkow and McLellan, 2016). Emergency department visits involving misuse or abuse of POs increased 1.5-fold between 2004 and 2011, while admissions to substance-abuse
treatment programs due to PO use increased more than 4-fold between 2002 and 2012 (Compton et al, 2016). Between 2000-2014, the US death rate from PO overdose increased from 1.5 to 5.9 per 100,000 persons, with a similar increase in death rate from heroin overdose alone, from 0.7 to 3.4 per 100,000 persons (Compton et al, 2016). In 2015, drug overdoses accounted for over 52 thousand deaths in the United States, 63% of which involved an opioid (Rudd et al, 2016); among these opioid-related deaths, 45% (approximately 15 thousand) involved a prescription opioid (Guy et al, 2017).

Effectively managing the opioid epidemic is difficult due to the utility of opioids in managing chronic pain; at any given time, between 30 – 40% of the US population reports experiencing acute or chronic pain (Wilson-Poe and Moron, 2017). When asked for the main reason for most recent opioid use, the majority of respondents (62.3%) report using POs to relieve physical pain, while significantly fewer (12.9%) reported use to feel good or get high (NSDUH, 2017). The source of obtaining POs also ranges; while a proportion of individuals in the US who misused POs in 2016 had them prescribed by a doctor (35.4%), more than half obtained POs from a friend or relative (53%), and often for free (40%) (NSDUH, 2017). Additionally, due to various factors in PO supply and price, dangers linked to the seeking of alternative opioids (e.g. heroin) have emerged. PO users are 40-fold more likely to become heroin users, and in both Canada and the US, spikes in heroin use have been observed when PO availability was curtailed by selective PO intervention (e.g. descheduling of specific POs, restricting prescribing guidelines, etc.) (Compton et al, 2016; Dart et al, 2015; Fischer and Rehm, 2017; Jones, 2013; Lake et al, 2016). In 2016, roughly 950 thousand individuals in the US had used heroin, while approximately 67% of those used POs and heroin concomitantly (NSDUH, 2017). More recently, the trajectory of PO abuse has led to seeking illicit PO analogs, such as fentanyl, as well as the lacing of POs with these illicit analogs, creating an additional aspect to the opioid epidemic (Fischer and Rehm, 2017). Over a six-month period in 2014 in one Florida Medical Examiner District, 97% of the overdose deaths reported involved accidental drug intoxication with fentanyl as a sole or contributing factor (Lee et al, 2016).

The increase in opioid abuse over the last two decades appears to predominate in higher socio-economic countries like Canada and the US (Fischer and Rehm, 2017; Fischer et al, 2006). Canada and the US account for roughly 80% of world PO consumption (Figures 3 and 4) (NSDUH, 2017).
This is due in part to POs being unavailable in most less-resourced world countries. However, this may also be due to the high availability of, and ease of access to, POs in North America, in which the medical system may rely more heavily on pharmaceutical treatments (Fischer and Rehm, 2017; Lohman et al, 2010). For example, in looking at commonly prescribed opioid compounds (often in combination with anti-inflammatories; see section 1.1.2), 10.4% of 26.5 million codeine users and 14.1% of 27.6 million oxycodone users reported misuse within the last year (Figure 5). There is a strong correlation between the amount of medical dispensing of POs and harmful PO-related outcomes (e.g. morbidity and mortality) (Wisniewski et al, 2008).

![Graph showing region-specific percentage of daily opioid consumption in 2001-2003, compared to 2011-2013. Adapted from the 2016 International Narcotics Control Board (INCB) availability report.](image)

**Figure 3:** Region-specific percentage of daily opioid consumption in 2001-2003, compared to 2011-2013. Adapted from the 2016 International Narcotics Control Board (INCB) availability report.
Figure 4: Country-specific opioid consumption per capita, and the prevalence of opioid misuse, in 2016. Adapted from the 2016 International Narcotics Control Board (INCB) availability report.

Figure 5: Total opioid use and reported misuse in individuals above the age of 12 in the US in 2016, divided by primary opioid compound. Axis labels: OC, Oxycodone; OM, Oxymorphone; COD, Codeine; MOR, Morphine; HC, Hydrocodone; HM, Hydromorphone; FEN, Fentanyl; TRAM, Tramadol; DEM, Demerol; MD, Methadone. Adapted from the National Survey on Drug Use History, detailed tables from 2016.
One consideration in addressing the epidemic, and balancing the care and needs of pain patients, is the spread of economic burden resulting from PO overdose, abuse, and dependence. In 2013, the aggregate economic burden of PO abuse and dependence in the US was estimated to be between 70 to 87 billion dollars (Florence et al, 2016). Within this estimate, non-fatal cases accounted for almost two thirds of the costs in health care ($26 billion, 33%), substance abuse treatment ($3 billion, 4%), and lost productivity ($20 billion, 26%) (Florence et al, 2016). Fatal cases accounted for a little more than one quarter of the costs (specifically in lost productivity and health care costs; $21 billion, 27%), while criminal justice (police protection, correctional facilities, legal fees) accounted for the last 10% of economic burden ($8 billion) (Florence et al, 2016). Quantifying some of the adverse health and economic impacts associated with POs may help decision makers select where to focus for mitigating future, and treating current, opioid dependence, to reduce the productive life-years lost to PO morbidity and mortality.

1.2 – CYPs
1.2.1 General introduction
Cytochrome P450 (CYP) enzymes are a superfamily of membrane-bound hemoproteins, responsible for metabolizing xenobiotic and endogenous compounds (Cook et al, 2016; Miksys and Tyndale, 2013). CYP-mediated metabolism is a complex, multistep process involving oxidation of a substrate after binding to the active site in the heme domain (Cook et al, 2016). Molecular oxygen is activated and incorporated into the CYP substrate, forming water as a by-product; this mechanism of oxidation is the basis for numerous reactions including hydroxylation, epoxidation, deamination, and dealkylation (Hannemann et al, 2007). CYP-mediated metabolism generally produces a hydrophilic metabolite, which is more easily excreted from the human body (Zanger and Schwab, 2013). These metabolites may have decreased (inactivation), increased (activation), or un-altered, pharmacologic activity compared to the parent compound. CYP metabolism of drugs occurs predominantly in the liver, where CYPs are highly expressed and are largely responsible for systemic drug and metabolite levels.

CYPs are divided into families, based on amino acid sequence homology (Nelson, 2006). While the majority of human CYPs function to maintain endogenous compound homeostasis (e.g. biosynthesis of steroid hormones, prostaglandins, bile acids, etc.), enzymes belonging to CYP
families 1-3 metabolize a large proportion of clinically indicated drugs and xenobiotics (Zanger and Schwab, 2013). For example, over 30% of clinically used drugs are metabolized by CYP3A4 (e.g. atorvastatin), while 20% are metabolized by CYP2D6 (e.g. dextromethorphan), 12.8% by CYP2C9 (e.g. diclofenac), 8.9% by CYP1A2 (e.g. caffeine), and 7.2% by CYP2B6 (e.g. bupropion) (Zanger and Schwab, 2013).

While CYP metabolism primarily occurs in the liver, extrahepatic (e.g. in the brain) CYP expression may alter drug activation/inactivation within a tissue/organ and thereby drug response. A large proportion of CNS-acting drugs are metabolized by CYP enzymes, particularly by the CYP2 family, to active and/or inactive metabolites (Zanger et al, 2004). Therefore, target-tissue drug metabolism in the brain may impact CNS-acting drug response contributing, in part, to why peripheral drug and/or metabolite levels do not always correlate to CNS-acting drug response (discussed further in 1.4) (Michels and Marzuk, 1993; Miksys and Tyndale, 2013).

1.2.2 CYP2D6 enzyme
1.2.2.1 Substrates and clinical drugs of interest
CYP2D6 is an important enzyme, as it metabolizes approximately 20% of clinically used drugs (Zanger et al, 2004). CYP2D6 shows a high degree of interindividual variability, primarily due to genetic polymorphisms, resulting in altered therapeutic consequences for CYP2D6 substrates (more in section 1.2.2.2). The enzyme was first characterized in humans as a consequence of interindividual variability in drug response. An inherited monogenic trait resulting in differing interindividual response to sparteine (an antiarrhythmic) and debrisoquine (an antihypertensive), referred to as the sparteine/debrisoquine polymorphism, was subsequently demonstrated to be due to a protein of the human CYP2D subfamily (Eichelbaum et al, 1987; Gonzalez et al, 1988). The CYP2D gene locus spans 45 kilobases on chromosome 22 (22q13.1) and contains three distinct genes: CYP2D6, CYP2D7 (shares sequence homology with CYP2D6 except for a base insertion in exon 1), and CYP2D8 (a pseudogene with sequence variations that prevent the formation of functional protein) (Gaedigk, 2013).

Substrates of CYP2D6 show structural similarities, including a planar hydrophobic aromatic ring and a nitrogen atom at a distance of about 5-7 Å from the site of oxidation, therefore initial
pharmacophore models using these arrangements were developed to predict compounds likely to be substrates (Zanger et al, 2004). Crystal structures of CYP2D6 with and without substrate bound have been developed for improving understanding of the active site architecture of the enzyme and have employed more accurate computational approaches to examine substrate binding (Rowland et al, 2006; Wang et al, 2012).

CYP2D6 metabolizes numerous xenobiotic compounds, including drugs that target the CNS and cardiovascular systems, as well as endogenous compounds. This includes antidepressants (e.g. fluoxetine), antipsychotics (e.g. haloperidol), analgesics (e.g. codeine), antiarrhythmics (e.g. propafenone), β-blockers (e.g. propranolol), amphetamines (e.g. 3,4-methylenedioxymethamphetamine), plant alkaloids (e.g ibogaine), neurotoxins (e.g. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP]), and endogenous neurochemicals (e.g. tyramine, 5-methoxytryptamine) (Table 1) (Miksys and Tyndale, 2013; Mo et al, 2009).
Table 1: Examples of CYP2D6 substrates and the reactions catalyzed by CYP2D6.

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<thead>
<tr>
<th>CYP2D6 Substrate</th>
<th>Reaction catalyzed by CYP2D6</th>
<th>CYP2D6 Substrate</th>
<th>Reaction catalyzed by CYP2D6</th>
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<td>5-methoxytryptamine</td>
<td>Demethylation</td>
</tr>
<tr>
<td>Promethazine</td>
<td>Hydroxylation</td>
<td>Tyramine</td>
<td>Demethylation</td>
</tr>
</tbody>
</table>

Adapted from Zanger et al. 2004 and Miksys and Tyndale 2013.

1.2.2.2 Variation in CYP2D6
1.2.2.2.1 Genetic sources of variation
The majority of CYP2D6 allelic variants are characterized by the presence of one or more nucleotide variations, including single nucleotide polymorphisms (SNPs), insertions, and deletions (Gaedigk, 2013). To date, over 80 sequence variants have been identified in CYP2D6 resulting in completely inactive function, reduced function, increased function (via additional functional gene copies), or no influence on the function of CYP2D6 enzyme activity (https://www.pharmvar.org/gene/CYP2D6). For example, the CYP2D6*10 variant allele contains
a mutation in a sequence necessary for folding of the CYP2D6 protein, therefore results in reduced protein stability and function (Ingelman-Sundberg, 2005). Gene duplications occur when individuals express multiple CYP2D6 copies on the same allele, with the majority being CYP2D6*2 (2850C>T and 4180G>C SNPs) deemed CYP2D6*2xN (with N representing the number of additional gene copies) (Aklillu et al., 1996; Dahl et al., 1995; Lundqvist et al., 1999). CYP2D6*1, *2, and *4 are the most common alleles which are duplicated, but duplication events occur in numerous other functional, non-functional, and reduced function alleles (Figure 6) (Gaedigk, 2013; Lundqvist et al., 1999).

**Figure 6: Genomic impact of select CYP2D6 variant alleles.** Predicted structures and functional impacts of multiple CYP2D6 alleles; the DNA impacts of mutations (green circles) are shown above the alleles and the amino acid impacts below, while open reading frames are indicated by grey shaded boxes. DNA abbreviations: A, adenosine; C, cytidine; G, guanosine; T, thymidine. Amino acid abbreviations: H, histidine; L, leucine; M, methionine; P, proline; R, arginine; S, serine; T, threonine. Adapted from Zanger et al. 2004.
CYP2D6 substrates primarily (>80%) metabolized by CYP2D6, such as debrisoquine or dextromethorphan, have been used for in vivo phenotyping of individuals into distinct CYP2D6 activity groups (Zanger et al, 2004). These phenotype groups, in order of ascending activity, are poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM), and ultra-rapid metabolizers (UM) (Zanger et al, 2004). Translating genotypes into clinically useful information is difficult, owing to the potentially hundred(s) of genotype combinations (diplotypes) that could exist in a population and due to overlap in phenotypic grouping. One cohort of 672 individuals was genotyped to have 95 different CYP2D6 diplotypes (Gaedigk et al, 2008), while over 100 unique diplotypes were identified in the first 800 individuals enrolled in the PG4KDS study (Hoffmann et al, 2014). Attempts have been made to better categorize CYP2D6 phenotypes from different genotypes, such as assigning an activity score to each allele (null: 0, reduced: 0.5, normal: 1, or increased: 2) and using the sum diplotype activity score as a metric of CYP2D6 activity (Gaedigk and Coetsee, 2008; Gaedigk et al, 2008).

1.2.2.1.1. Interethnic variability

The frequencies of CYP2D6 polymorphisms and resulting phenotype groups vary across ethnicities as specific CYP2D6 variant alleles are found at higher and lower frequencies in different populations. White European populations have a high incidence of PMs at 3-10% of the total population, while Black Africans and African Americans range between 2-7% and East Asian populations between 0-2% (Teh and Bertilsson, 2012). This is due, in part, to the higher frequency of null-function CYP2D6*3, *4, *5, *6, and reduced-function *10 and *41 alleles in White European populations, with low frequencies of CYP2D6*3 and *4 alleles in East Asian and Oceania populations (Gaedigk, 2013; Teh and Bertilsson, 2012). CYP2D6*36 contributes substantially to the PM status in Asian (3% allele frequency) and African American (0.5%) populations (Gaedigk et al, 2006). The frequencies of common CYP2D6 variant alleles are displayed for select ethnicities in Figure 7.
Figure 7: Select CYP2D6 allele frequencies across ethnic groups. Combined results of 172 independent studies. Black: Black Africans and African Americans; Asian: South Asians and East Asians; Native: Native Oceanians, Native Americans, and Circumpolar Populations; White: Caucasian American, Scandinavian, European, Mediterranean, and Slavs. Adapted from Llerena et al. 2014.

The reduced function CYP2D6*10 allele is the most common variant allele in Asians; in Chinese, CYP2D6*10 comprises 51-70% of all CYP2D6 alleles (Johansson et al, 1994; Johansson et al, 1991). This may account for the lower overall mean CYP2D6 enzyme activity in Asians compared to Caucasians, as well as an overall rightward shift in the median CYP2D6 metabolic ratio in Chinese EMs (Johansson et al, 1994; Johansson et al, 1991). Europeans have the lowest frequency of CYP2D6*10 at 1-2%, while the overall frequency in other populations ranges from 3-7% (Gaedigk, 2013). Black African and African American populations have a high frequency of the CYP2D6*17 allele (14-26%), which contains two missense mutations (850C>T SNP and 4180G>C) resulting in an altered CYP2D6 active site (Gaedigk, 2013; Teh and Bertilsson, 2012). The CYP2D6*17 allele is also found among other populations with African founders such as Brazilians (Kohlrausch et al, 2009), Cubans (Llerena et al, 2012), Trinidadians (Montane Jaime et al, 2013), and South Africans (Dodgen et al, 2013; Wright et al, 2010).

The percentage of CYP2D6 gene duplications or multiplications, resulting in CYP2D6 UMs, also varies across populations. The allele frequency of CYP2D6*1xN or *2xN in European populations
is around 2% (e.g. 1-2% in Swedish Caucasians) which is comparative to Japanese, Chinese, and Indian populations (Aklillu et al., 1996; Kubota et al., 1996; Qin et al., 2008). Frequencies of CYP2D6 duplications are higher in Mediterranean (e.g. 7-10% in Spanish) and North African (e.g. 29% in black Ethiopians) populations (Aklillu et al., 1996; Teh and Bertilsson, 2012).

1.2.2.2.1.2 Functional impact of CYP2D6 polymorphism

CYP2D6 polymorphism results in a range of CYP2D6 enzyme activity which alters drug metabolism and response, drug efficacy (e.g. activation or inactivation), and adverse drug effects (e.g. toxicity). There is extensive evidence of individuals with different CYP2D6 phenotypes having altered response to psychotropic drugs (e.g. haloperidol), cardiovascular drugs (e.g. metoprolol), antineoplastics (e.g. tamoxifen), and analgesics (e.g. codeine, discussed further in 1.3) (Teh and Bertilsson, 2012). CYP2D6 genotype has also been associated with disease risk including for Alzheimer’s disease and Parkinson’s disease (Golab-Janowska et al., 2007).

Most antipsychotics, including haloperidol, are metabolized by CYP2D6. In a random sample of schizophrenic inpatients treated with haloperidol an increased number of active CYP2D6 genes correlated with increased haloperidol clearance and trended towards lower haloperidol therapeutic efficacy (Brockmoller et al., 2002; Teh and Bertilsson, 2012). The occurrence of pseudo-parkinsonism (a haloperidol-induced adverse reaction), among other extrapyramidal side effects, was higher in CYP2D6 PMs, likely due to the reduced metabolic clearance of haloperidol in these individuals (Brockmoller et al., 2002).

Metoprolol is a cardio-selective β-blocker used in the treatment of hypertension, angina pectoris, cardiac arrhythmia, and chronic heart failure; CYP2D6 is responsible for roughly 75% of the total metabolism of metoprolol through α-hydroxylation (10% of total dose) and O-demethylation (65%) (Borg et al., 1975; Prakash and Markham, 2000). Total plasma exposure to metoprolol is 4- to 6-fold higher in CYP2D6 PMs than EMs after a single dose, while there is a 10-fold difference in metoprolol clearance between PMs and UMs (Hamelin et al., 2000; Kirchheiner et al., 2004). PMs have a 2-fold greater metoprolol-induced cardiovascular effect (reduced exercise-induced heart rate) than UMs, and they have a 5-fold higher risk of developing adverse effects to metoprolol (Kirchheiner et al., 2004; Wuttke et al., 2002).
Tamoxifen is a selective estrogen receptor modulator used as a treatment for breast cancer (Schroth et al., 2009). Tamoxifen is metabolized into an active metabolite endoxifen by N-demethylation and 4-hydroxylation carried out by CYP2D6 (Schroth et al., 2009). Individuals homozygous for the null-function CYP2D6*4 and taking tamoxifen to treat estrogen receptor-positive breast cancer have significantly decreased relapse-free survival compared to those heterozygous or homozygous for CYP2D6*1 (wild-type) (Schroth et al., 2009). Similarly, Japanese women with breast cancer homozygous for the reduced-function CYP2D6*10 had lower steady state plasma levels of endoxifen from tamoxifen, shorter median time to disease, and higher incidence of breast cancer recurrence within 10 years (Kiyotani et al., 2008; Lim et al., 2007; Lim et al., 2011; Xu et al., 2008). While routine testing of women on tamoxifen has not been approved for risk stratification, numerous reports support CYP2D6 genotype analysis being used as a predictive biomarker for treatment success (Gaedigk and Coetsee, 2008).

1.2.2.2 Non-genetic sources of variation and drug-drug interactions

Beyond genetic polymorphism, non-genetic variation in CYP2D6 protein level can also occur. Hepatic CYP2D6 displays constitutive expression during adulthood and is therefore relatively unaltered by common CYP inducers like phenobarbital (Edwards et al., 2003; Stevens et al., 2008). However, the CYP2D6 gene can be regulated by binding of transcription factors to the CYP2D6 promoter region, such as hepatocyte nuclear factor (HNF) 4α (Pan et al., 2017). In a human liver cell line (HepG2), binding of HNF4α enhanced promoter activity of CYP2D6; HNF4α mRNA expression correlated with microsomal CYP2D6 enzyme activity (Cairns et al., 1996; Yang et al., 2010). HNF4α knockdown decreased CYP2D6 expression in human hepatocytes, indicating that HNF4α is required for constitutive CYP2D6 activity (Jover et al., 2001). Similarly, in a HepG2 cell line, overexpression of the transcription factor CAAT/enhancer-binding protein α increased CYP2D6 mRNA (Jover et al., 1998; Pan et al., 2017).

Since CYP2D6 metabolizes a large portion of clinically used drugs, drug-drug interactions can occur through competition or inhibition by a CYP2D6 substrate or inhibitor (Table 2). Inhibition can occur by competitive inhibition (binding of an inhibitor in or near the enzyme active site blocking substrate accessibility), irreversible inhibition (metabolism of a mechanism-based
inhibitor (MBI) by CYP2D6 to a reactive intermediate that binds irreversibly to the enzyme rendering it inactive), non-competitive inhibition (binding of an inhibitor to the enzyme away from the active site, altering the conformation of the enzyme), or uncompetitive inhibition (binding of an inhibitor to the enzyme after substrate has bound) (Bertelsen et al, 2003; Lin and Lu, 1998). For example, propranolol, a non-selective β-adrenergic receptor blocker, is a MBI of CYP2D6; propranolol is hydroxylated by CYP2D6 to 4-hydroxypropranolol which forms a reactive intermediate that binds to, and inactivates, CYP2D6 (Rowland et al, 1994). Paroxetine, a selective serotonin reuptake inhibitor, is also a potent MBI of CYP2D6 (Bertelsen et al, 2003). In schizophrenic patients receiving risperidone (anti-psychotic and CYP2D substrate), paroxetine dose-dependently increased plasma risperidone concentrations and associated extra-pyramidal side effects (Saito et al, 2005). By comparison, quinidine is a potent and selective competitive inhibitor of CYP2D6, but not a substrate itself (Branch et al, 2000; McLaughlin et al, 2005).

1.2.2.3 Species differences in CYP2D6 enzyme
CYP2D enzymes have been identified in numerous species including humans, rat, mouse, and monkey. While humans have one functional isoform of CYP2D (CYP2D6) rats have six (CYP2D1, 2, 3, 4, 5, and 18) with unique substrate specificity, metabolism, expression, and inhibition profiles (Hiroi et al, 2002). For example, while rat CYP2D1 and CYP2D2 are the most abundant isoforms in the liver, CYP2D4 is primarily expressed in rat brain (Wyss et al, 1995). However, rat CYP2D enzymes collectively, are a useful model of human CYP2D, due to relatively similar amino acid sequence identity (>70% for all isoforms) and the ability to perform metabolic reactions in vitro similar to human CYP2D6 (Venhorst et al, 2003). Human CYP2D6 and rat CYP2D2 exhibit high levels of 4-hydroxylation of debrisoquine and propranolol (Hiroi et al, 2002). Similarly, human CYP2D6 and rat CYP2D2 (along with rat CYP2D1) O-demethylate codeine to morphine in vitro (Grobe et al, 2012).
Table 2: Examples of competitive and mechanism-based CYP2D6 inhibitors.

<table>
<thead>
<tr>
<th>DRUG CLASS</th>
<th>INHIBITOR</th>
<th>DRUG CLASS</th>
<th>INHIBITOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiarrhythmics</td>
<td>Propafenone</td>
<td>Antiarrhythmics</td>
<td>Desethylamiodarone</td>
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<tr>
<td></td>
<td>Quinidine</td>
<td>Antidepressants</td>
<td>Paroxetine</td>
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<tr>
<td>Antidepressants</td>
<td>Citalopram</td>
<td>Antiemetics</td>
<td>Metoclopramide</td>
</tr>
<tr>
<td></td>
<td>Desipramine</td>
<td>Antihistamines</td>
<td>Cimetidine</td>
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<tr>
<td></td>
<td>Fluoxetine</td>
<td>Antipsychotics</td>
<td>Pimozide</td>
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<tr>
<td></td>
<td>Imipramine</td>
<td>β-adrenergic Blockers</td>
<td>Propranolol</td>
</tr>
<tr>
<td></td>
<td>Mirtazapine</td>
<td>Recreational Drugs</td>
<td>3,4-methylenedioxy-methamphetamine</td>
</tr>
<tr>
<td>Antipsychotics</td>
<td>Haloperidol</td>
<td></td>
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<tr>
<td></td>
<td>Thioridazine</td>
<td></td>
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<tr>
<td>β-adrenergic Blockers</td>
<td>Metoprolol</td>
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<td></td>
<td>Timolol</td>
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</table>


1.3 – CYP2D6 and opioids
1.3.1 CYP2D6 metabolism of opioids

CYP2D6 plays an important role in metabolizing opioids. This is due to 1) CYP2D6 metabolism of opioids producing metabolites that are more potent in activating ORs, 2) *CYP2D6* polymorphisms altering the formation of these metabolites and thereby altering therapeutic effect or toxicity, and 3) other CYP2D6 substrates or inhibitors interacting with opioid metabolism (Smith, 2009, 2011). For these reasons, genetic and non-genetic variability in CYP2D6 activity have been studied in relation to inter-individual variation in opioid response, side effects, tolerance, and abuse liability.
While usually a minor metabolic pathway, the O-demethylation of opioids by CYP2D6 generally produces a metabolite with greater OR affinity (Figure 8). This may result in a CYP2D6 metabolite that modestly contributes to (e.g. hydrocodone to hydromorphone), or is entirely responsible for (e.g. codeine to morphine), analgesia (Chen et al, 1991a; Grond and Sablotzki, 2004; Smith, 2009). While the CYP2D6-mediated metabolism of codeine to morphine represents a relatively minor pathway of codeine metabolism (0-15% of urinary recovery) morphine has a 3000-fold greater affinity for the MOR than codeine; therefore, codeine analgesia depends on this metabolic activation (Adler et al, 1955; Chen et al, 1991b; Pert and Snyder, 1973). While a similar percentage of oxycodone is metabolized by CYP2D6 (11% of urinary recovery) to oxymorphone, a 44-fold more potent MOR activating metabolite (9-fold greater than morphine), oxycodone itself also has a relatively high affinity to MOR (only 5-fold less than morphine) and therefore, unlike codeine, oxycodone does not require CYP2D6 activation to elicit analgesia (Klimas et al, 2013; Lalovic et al, 2006).

1.3.2 Impact of CYP2D6 phenotype on opioid analgesia
The wide range of CYP2D6 metabolism phenotypes across populations makes it imperative to understand the relative consequences on opioid metabolism and response. For active parent opioid compounds primarily metabolized by CYP2D6, a lower clearance in PMs may produce higher plasma concentrations of the parent compound and thereby more side-effects (Zahari and Ismail, 2014). Conversely, UMs may experience no or less pain relief leading to therapeutic failure (Zahari and Ismail, 2014). For pro-drug opioids that require bioactivation by CYP2D6 to active metabolites to elicit therapeutic effect (e.g. codeine to morphine) PMs may produce no CYP2D6-mediated metabolite and therefore experience no analgesia, while UMs may form metabolite in excess and therefore experience more pronounced analgesia and a greater likelihood of opioid-related side effects (Zahari and Ismail, 2014).
Figure 8: Structures and properties of opioid parent compounds and CYP2D6-mediated metabolites. Codeine, oxycodone, and hydrocodone (A) structures differ by defined functional groups ($R_1$ and $R_2$), while tramadol (B) has a unique structure.

Codeine analgesia is solely mediated by the CYP2D6 formation of morphine and is therefore often used as a prototypical example of the impact of CYP2D6 genotype on opioid response. In general, CYP2D6 PMs produce little-to-no morphine from codeine, with extremely low plasma morphine concentrations, and therefore little-to-no analgesia while UMs may experience more severe side-effects, likely mediated by greater morphine concentrations (Kirchheiner et al., 2007). In healthy volunteers given codeine, CYP2D6 PMs had 29-fold lower maximum plasma morphine concentration than EMs and had no analgesic effect, tested as a response to selective transcutaneous nerve stimulation (Desmeules et al., 1991). Similarly in another set of healthy volunteers given codeine, CYP2D6 PMs had a 15-fold lower plasma morphine AUC and 11-fold lower total excreted morphine than EMs, and reduced effects of codeine on gastrointestinal motility (Mikus et al., 1997). Inhibiting CYP2D6 with quinidine in EMs resulted in undetectable plasma morphine after codeine and no change in pin-prick pain thresholds compared to placebo, thus inhibition abolished morphine formation and codeine effect (Sindrup et al., 1992).
Tramadol is metabolized by CYP2D6 to a 300-fold more pharmacologically active metabolite O-desmethyltramadol (Figure 8) (Abdel-Rahman et al., 2002). In patients receiving tramadol for post-operative analgesia, the mean O-desmethyltramadol plasma concentrations were lower in individuals with a CYP2D6 PM phenotype (0 ng*h/ml AUC) compared to UM (149.7 ng*h/ml), and were lower in individuals co-medicated with CYP2D6 inhibitors (Stamer et al., 2007). Consequently, more CYP2D6 PMs (81%) were categorized as tramadol non-responders compared to patients with all other phenotypes (10-20%); PMs also required more tramadol analgesics (calculated by total consumed mg of tramadol) over 48 h after surgery (Stamer et al., 2007). In a Chinese population receiving tramadol post elective nephrectomy, individuals homozygous for the reduced function CYP2D6*10 allele reported higher post-operative pain (VAS scores) and required more tramadol than individuals with heterozygous and wild-type (*1/*1) genotypes (Dong et al., 2015).

In some cases, although CYP2D6 metabolizes an opioid to a more pharmacologically active metabolite, CYP2D6 genotype does not affect response. In post-operative patients receiving oxycodone, plasma oxymorphone concentrations and the CYP2D6-metabolic ratio (oxymorphone/oxycodeone) differed across CYP2D6 phenotypes and were greater in CYP2D6 UMs than PMs; however pain scores did not change (Stamer et al., 2013). When CYP2D6 was inhibited by quinidine in healthy CYP2D6 EMs, oxymorphone levels were undetectable in 80% of the subjects however the subjective effects of oxycodone were unaltered (Heiskanen et al., 1998). Similarly, inhibiting CYP2D6 in healthy subjects with paroxetine reduced the oxymorphone AUC by 44%, and increased the CYP3A4-mediated metabolite noroxycodone AUC by 68%, but did not alter subjective effects of oxycodone (Gronlund et al., 2010). The analgesic response to hydrocodone, a 6-keto opioid with similar structure to oxycodone, appears to be similarly unaffected by CYP2D6 phenotype (Yiannakopoulou, 2015). In a large prospective observational study, individuals taking at least one CYP2D6 substrate or inhibitor experienced less hydrocodone analgesia than those without (1.7-fold lower change in VAS score) (Monte et al., 2014). However, a separate study showed that CYP2D6 EMs with active CYP2D6, EMs with inhibited CYP2D6 (via quinidine), and CYP2D6 PMs have no difference in subjective response to hydrocodone, suggesting minimal role of CYP2D6 metabolism in hydrocodone analgesia (Kaplan et al., 1997).
1.3.3 Impact of CYP2D6 phenotype on opioid abuse liability

There are numerous pharmacologic characteristics of opioid response that contribute to abuse potential including, but not exclusive to, speed of onset of drug response, duration of response, and net positive (e.g. analgesia and euphoria) and negative (e.g. adverse events) effects (Wightman et al, 2012). Like analgesia, the subjective effects (e.g. euphoria and elation) of opioids are mediated by action at MORs. Therefore, interindividual differences in CYP2D6 phenotype, resulting in differences in the formation of metabolites that influence positive and negative subjective effects, may be useful to predict opioid-specific abuse potential. In support of this, CYP2D6 PMs were under-represented in individuals dependent on oral opioids (0% of 83 individuals), compared to those with multi-drug dependencies (4% of 93) and with no drug dependencies (6.5% of 93) (Tyndale et al, 1997).

Codeine exhibits pronounced interindividual variation in analgesia, due to wide variation in CYP2D6 phenotype resulting in altered formation of the active metabolite morphine (Desmeules et al, 1991). Similarly, variation in this CYP2D6 activation alters codeine abuse potential. In healthy non-drug-dependent CYP2D6 EMs inhibiting CYP2D6, with single and short-term quinidine treatment, decreased morphine formation and positive subjective effects from a preferred dose of codeine (Kathiramalainathan et al, 2000).

Assessing individual CYP2D6 phenotype may be able to predict oxycodone’s abuse potential, despite the more active CYP2D6 metabolite of oxycodone, oxymorphone, not contributing significantly to oxycodone analgesia. Predictive pharmacogenetic/pharmacokinetic models have been used to predict oxycodone abuse potential from CYP2D6 phenotype (Linares et al, 2014). However, analgesia models show no significant contribution of the CYP2D6 metabolite of oxycodone, oxymorphone, to oxycodone analgesia, therefore the validity of these models is unknown. In healthy CYP2D6 EMs and PMs, there were no significant differences in objective (e.g. pupil dilation) and subjective (e.g. Addiction Research Center Inventory scale) effects elicited by hydrocodone (Kaplan et al, 1997). Inhibiting CYP2D6 with quinidine abolished the pharmacokinetic difference between EMs and PMs, determined by the parent (hydrocodone) to metabolite (hydromorphone) ratio, but had no impact on hydrocodone subjective effects,
suggesting CYP2D6 activity does not contribute to hydrocodone’s abuse potential (Kaplan et al., 1997).

1.3.4 Other sources of variability in opioid response
Beyond genetic variation in the CYP2D6 metabolism of opioids, there are numerous additional sources of interindividual variation in opioid response, including variation in OR expression and function, metabolism by enzymes other than CYP2D6, and extrahepatic metabolism of opioids. Variants in OPRM1, the gene encoding the MOR, may influence opioid response in humans by altering receptor function or substrate binding. The 802T>C OPRM1 SNP is located in an intracellular protein domain and interferes with G-protein coupling and receptor desensitization, resulting in diminished MOR potency of DAMGO, β-endorphin, and morphine (Lotsch et al., 2004). However, the 802C allele is rare across populations (allele frequency <1%) which has made study of the functional outcome in humans challenging (Befort et al., 2001; Koch et al., 2000). In comparison, the 118A>G OPRM1 SNP is located on an extracellular binding region, altering substrate binding, and has relatively high frequencies (allele frequency of 10 – 50% across ethnicities) (Mague and Blendy, 2010). Individuals heterozygous or homozygous for the 118G OPRM1 variant experience reduced analgesic response to morphine and numerous other opioids (Mague and Blendy, 2010).

CYP3A4 represents a major metabolic pathway for numerous opioids (Lotsch et al., 2004). Due to a large number of drug compounds metabolized by CYP3A4, clinically significant drug-drug interactions may occur between opioids and CYP3A4 substrates. The anti-viral medications ritonavir and nevirapine induce the CYP3A4 metabolism of methadone, therefore decreasing its pharmacologic effect; HIV-patients receiving ritonavir and nevirapine alongside methadone treatment experience less methadone effect and therefore heightened opioid-withdrawal symptoms (Geletko and Erickson, 2000; Heelon and Meade, 1999). Genetic polymorphisms in the human CYP3A4 gene have been identified, however the clinical influence on opioid metabolism and response is currently unknown (Lotsch et al., 2004).

While interindividual variation in opioid metabolism has primarily focused on hepatic CYP metabolism altering circulating plasma opioid parent and/or metabolite levels, CYP-mediated drug
metabolism in extra-hepatic tissues, such as the brain, has emerged as an novel source of variation in drug response (see Appendix A) (McMillan and Tyndale, 2017a; Miksys and Tyndale, 2013). As the brain is the primary site of opioid action, local metabolism of opioid compounds may impact brain parent and metabolite levels and resulting therapeutic effects, independent of peripheral metabolism and systemic drug levels. This novel source of interindividual variation in opioid response is the primary focus of this thesis, and further discussed in the following introduction section 1.4.

1.4 – Brain CYPs
1.4.1 Brain CYP expression
CYPs are expressed in the brains of numerous species, where they may contribute to the local metabolism of exogenous and endogenous compounds, and to neuroprotection (Meyer et al, 2007). The majority of the types of human CYP mRNA transcripts found in the liver have been found in the brain, however only a small number of these isoforms have been studied at the level of protein and/or activity (Dutheil et al, 2009; Miksys and Tyndale, 2013). This is due, in part, to uncertainty as to whether an adequate environment exists (e.g. required cofactors and coenzymes) to allow brain CYP metabolism in vivo, and to total brain CYP content being low relative to liver (1–2%) (Hedlund et al, 2001; Miksys and Tyndale, 2013). The relative expression of CYPs in the brain, compared to the liver, varies across isoforms. CYP46A1 transcripts are virtually absent in the liver while highly expressed in the brain; CYP1B1 and CYP2U1 transcripts are expressed in the brain at twice the level of liver, and CYP2J2 levels in the brain are 10% that of liver (Agundez et al, 2014). Other isoforms such as CYP1A1, CYP3A4, and CYP2E1, while detected in human brain, are expressed at low transcript levels in normal conditions (Agundez et al, 2014). However, the cell- and region-specific expression of CYPs may produce microenvironments in the brain that may influence CNS-acting drug effects in those cells and/or regions (Britto and Wedlund, 1992).

Hepatic CYPs are generally expressed in the endoplasmic reticulum, while brain CYPs are found on mitochondrial membranes, the plasma membrane, and other cell compartment membranes, and display large variability in species and regional expression (Walther et al, 1986). Human CYP1A mRNA and protein have been identified in the cortex, thalamus, and numerous brainstem nuclei (e.g. substantia nigra, median raphe, and locus coeruleus) (Farin and Omiecinski, 1993; McFadyen
et al., 1998). Rat CYP1A mRNA and protein exhibit similarly high expression in the cortex, but also in the cerebellum, midbrain, and striatum (Schilter and Omiecinski, 1993). Human and rat CYP2B mRNA and protein exhibit high expression in the cortex, striatum, hippocampus, basal ganglia, and cerebellum, while rat brain CYP2B is further expressed in the olfactory bulbs and hypothalamus (Farin and Omiecinski, 1993; McFadyen et al., 1998; Miksys et al., 2003; Schilter and Omiecinski, 1993). Species differences in regional expression of brain CYPs is important to consider when relating the functional outcome of brain CYP metabolism in animal models of variable brain CYP metabolism to the potential impact in humans (further discussed in 1.4.3).

CYPs are also expressed at the BBB, an important endothelial barrier to compounds entering the CNS, where CYPs may act in conjunction with transport proteins to provide protection for the CNS (Agundez et al., 2014; Decleves et al., 2011). CYP1B1 and CYP2U1 mRNA in humans are highly expressed in cerebral micro-vessels at the BBB, as well as CYP2E1 and CYP2D6 (Dauchy et al., 2008). CYP2B may also function at the BBB interface as it is highly expressed in astrocytes with end-feet that envelop cerebral blood vessels (Miksys and Tyndale, 2013). The precise function of CYPs expressed at the BBB is currently unknown; however, their localization at this important juncture of xenobiotic permeation to the CNS suggests a role for protection against the entry of drugs or environmental toxins.

1.4.2 Brain CYP activity
After the discovery of brain CYP expression, it was unknown whether brain CYP enzymes were functional in vivo, or able to influence the levels and resulting effect of exogenous or endogenous compounds. Since parent and/or metabolite levels in the periphery, influenced by liver CYPs, can often cross the BBB it is difficult to separate the relative contribution of brain and liver CYPs to the levels of a specific metabolite in the brain (Miksys and Tyndale, 2013). Furthermore, in vitro study of brain CYP membranes is difficult, as their activity is sensitive to storage; 80% of rat brain CYP2D membrane activity is lost when stored in Tris buffer rather than artificial cerebrospinal fluid while 40% of rat brain CYP2D membrane activity is lost when kept at -30°C for one week (Tyndale et al., 1999). Nevertheless, in vitro studies in rat brain membranes confirmed the metabolism of a variety of CYP-substrates, such as the CYP2D-mediated hydroxylation of the
bufuralol or the CYP2B-mediated oxidation of parathion (Albores et al., 2001; Coleman et al., 2000).

Initial evidence of brain CYP activity in situ was investigated through using two mechanism-based inhibitors of rat brain CYP2B. [3H]-8-methoxypsoralen (8MOP), a radiolabelled CYP2B mechanism-based inhibitor, was injected into rat frontal cortex whereby, upon metabolism by CYP2B enzyme to a radiolabelled metabolite, 8MOP would inhibit and label CYP2B (Koenigs and Trager, 1998). CYP2B would only become radiolabelled if 8MOP were metabolized (i.e. if CYP2B was enzymatically functional); a monoclonal antibody was used to immunoprecipitate CYP2B and radiolabelled enzyme was detected (Miksys and Tyndale, 2009). The specificity of mechanism-based inactivation of CYP2B by 8MOP was assessed by pre-treating the frontal cortex unilaterally with another mechanism-based inhibitor of CYP2B, C8-Xanthate, prior to bilateral 8MOP treatment (Miksys and Tyndale, 2009; Yanev et al., 2000). The side of the frontal cortex pre-treated with C8-Xanthate had a reduced amount of 8MOP-radiolabelled immunoprotein compared to the contralateral side, providing further evidence that brain CYP2B was functional in vivo (Miksys and Tyndale, 2009). Similar techniques have been used to demonstrate a functional impact of brain CYP2B metabolism on CNS-acting CYP2B substrate drug response in vivo. Inhibiting brain-specific CYP2B in rat with a single intracerebroventricular injection of C8-Xanthate increased brain levels of the sedative-anesthetic propofol (inactivated by CYP2B) and increased propofol drug effect, measured by sleep time (Khokhar and Tyndale, 2011). There was no difference in ex vivo hepatic CYP2B metabolism between animals given intracerebroventricular vehicle or inhibitors (C8-Xanthate or 8MOP), indicating no effect on brain-administered CYP2B inhibitors on liver CYP2B activity (Khokhar and Tyndale, 2011). Evidence of the functional impact of brain CYP metabolism on drug response in experimental animals has now been demonstrated across different CYP isoforms (e.g. CYP2B and CYP2D) and CNS-acting drug classes (e.g. antipsychotics and neurotoxins), through novel techniques of brain-specific CYP inhibition and induction (more examples in 1.4.4.3 and 1.4.4.4) (Ferguson and Tyndale, 2011; Miksys and Tyndale, 2013). While brain CYP metabolism may not influence drug pharmacokinetics by way of peripheral metabolism and clearance, local metabolism in the brain may influence drug levels at the target site of action and have significant consequences on drug response.
1.4.3 Regulation of brain CYP activity

Brain CYPs differ from their hepatic CYP counterparts in their sensitivity to xenobiotic inducers in an isoform, brain-region, and mechanism specific manner (Miksys and Tyndale, 2013). Phenobarbital, a potent inducer of numerous hepatic CYPs, increases the expression and activity of brain and liver CYP2B1 and CYP2B2 in both rats and monkeys (Lee et al., 2006c; Schilter et al., 2000). Phenobarbital also increases brain CYP2E1 protein levels in rats and monkeys, but liver CYP2E1 protein and mRNA levels in rats but not monkeys (Caron et al., 2005a; Lee et al., 2006a). Nicotine and ethanol independently, and together, increase brain and liver CYP2E1 in monkeys and rats (Miksys and Tyndale, 2013). This is consistent with higher brain CYP2E1 levels observed in alcoholics and smokers compared to non-alcoholics and non-smokers (Ferguson et al., 2013; Howard et al., 2003; Joshi and Tyndale, 2006b; Warner and Gustafsson, 1994). However, nicotine and ethanol may regulate brain CYP2E1 via different mechanisms; for example, ethanol treatment induces rat brain CYP2E1 mRNA levels, suggesting a transcriptional mechanism of regulation, while nicotine induction of rat brain CYP2E1 does not, suggesting increased translational efficiency or protein stability (Howard et al., 2003; Joshi and Tyndale, 2006a; Zhong et al., 2012). The species and isoform specific effects of inducers on brain CYP expression, together or independent of an effect on liver CYP expression, is hallmark to brain CYP metabolism and important for understanding the translation of in vivo studies on their functional significance.

Short term nicotine pretreatment (seven days, subcutaneously) induces rat brain CYP2B, with no change in liver CYP2B protein and mRNA levels (Khokhar et al., 2010; Miksys et al., 2000a). Similarly, monkey brain CYP2B6 protein is induced by nicotine with no increase in mRNA or liver protein levels (Ferguson et al., 2013). Smokers have higher levels of CYP2B6 in the brain compared to non-smokers, as assessed in post-mortem brain samples, while liver CYP2B6 expression does not differ (Hesse et al., 2004; Miksys et al., 2003). This suggests that similar to the effects seen in rats and monkeys, nicotine, the primary psychoactive component in cigarettes, may induce CYP2B in human brain but not liver.

Brain CYP expression is further regulated by endogenous factors including hormonal and other factors associated with development (Miksys and Tyndale, 2013). Human liver CYP2D is constitutively expressed across an adult individual’s lifetime; however, brain CYP2D6 levels
(assessed in the frontal cortex) increase with age (Mann et al, 2012; Treluyer et al, 1991). Sex-based differences may also exist in brain CYP expression; exogenously administered estrogen and testosterone increases, while progesterone decreases, brain CYP2D expression and mRNA levels in female rats (Baum and Strobel, 1997; Bergh and Strobel, 1996). Transcripts for nuclear receptors have also been detected in the brain (e.g. peroxisome proliferator-activated receptor and retinoid X receptor), that may regulate the expression of brain CYPs in a similar, or different, manner to liver CYPs (Nishimura et al, 2004; Petersen et al, 2000). The overall extent of endogenous regulation of brain CYP expression, and impact in vivo, is still relatively unknown and poorly understood.

1.4.4 Brain CYP2D

1.4.4.1 Brain CYP2D expression and activity

CYP2D enzyme has been identified in the brains of numerous species, including rat, mouse, dog, monkey, and human (Miksys et al, 2002; Miksys et al, 2005; Siegle et al, 2001; Tyndale et al, 1999). CYP2D6, in humans, is expressed in the brain at levels that are a fraction of liver; however, is one of the most prominent drug metabolizing CYPs expressed in the brain (Dutheil et al, 2009). Human CYP2D6 is expressed heterogeneously across most brain regions, such as in the cortex, nucleus accumbens, hippocampus, and thalamus, with highest expression in the caudate, putamen, cortex, and cerebellum (Miksys et al, 2002). Brain CYP2D6 expression is cell-specific; for example, CYP2D6 immunoreactivity was detected in neurons of the substantia nigra, pyramidal cells in the frontal cortex, Purkinje and glial cells in the cerebellum, and endothelial cells at the BBB (Dutheil et al, 2009; Dutheil et al, 2010; Miksys et al, 2002). The region and cell specific expression of brain CYP2D is relatively consistent across species, with marked similarities in regions with highest brain CYP2D expression. Rat brain CYP2D is expressed heterogeneously with highest levels in the cerebellum, frontal cortex, parietal cortex, pons, medulla, and spinal cord (Miksys et al, 2000b). Similar to human brain CYP2D6, rat brain CYP2D showed strong immunoreactivity in neurons of the substantia nigra, pyramidal neurons in the neocortex, Purkinje cells in the cerebellum, and glial cells, but also in neurons in the olfactory bulb and pons, and endothelial cells in the choroid plexus (Miksys et al, 2000b; Miksys et al, 2005; Norris et al, 1996). Rat CYP2D4 and CYP2D18 are highly expressed in the brain; however, of the six rat CYP2D
isozymes, the relative region- and cell-specific expression of each enzyme is unknown and difficult to discern (Komori, 1993; Miksys et al, 2000b; Wyss et al, 1995).

The activity of brain CYP2D was first demonstrated in vitro by the ability of rat brain microsomes to perform the same enzymatic conversions as human or rat liver CYP2D; for example, rat brain membranes catalyzed the demethylation of codeine and dextromethorphan, the demethylation of 3,4-methylenedioxyxymethamphetamine, and the hydroxylation of bufuralol (Chen et al, 1990; Coleman et al, 2000; Jolivalt et al, 1995; Lin et al, 1992). Different rat brain regions metabolized dextromethorphan to varying degrees, with the highest catalytic activity observed in cerebellar, olfactory, and hippocampal membranes, notably, regions of highest CYP2D mRNA and protein expression (Miksys et al, 2000b; Tyndale et al, 1999). Brain membranes also biosynthesized the monoaminergic neurotransmitters dopamine and serotonin in vitro (Hiroi et al, 1998b; Yu et al, 2003). Formation of dopamine from tyramine and serotonin from 5-methyltryptamine was demonstrated in rat brain membranes, with highest formation observed, of regions tested, in the cerebellum; this was inhibited in vitro by the CYP2D inhibitor quinine and anti-CYP2D4 antibodies (Bromek et al, 2010; Haduch et al, 2013). The activity of brain CYP2D in situ was later tested by assessing CYP2D-mediated metabolite levels directly in the brain. When rats were given an intraperitoneal dose of codeine and morphine that resulted in similar plasma morphine concentrations at 30 min post-injection, ex vivo brain morphine levels were only detectable after codeine, suggesting local formation from codeine by brain CYP2D (discussed further in 1.4.4.4) (Chen et al, 1990).

Recently, the in vivo activity of brain CYP2D was demonstrated using a CYP2D mechanism-based inhibitor propranolol, similar to the demonstration of brain CYP2B activity in vivo. Propranolol is hydroxylated by CYP2D to a reactive metabolite that binds the active site of the enzyme, thereby inactivating it (Narimatsu et al, 2001). Administering propranolol intracerebroventricularly to rats, 24 hours prior to testing, yielded decreased ex vivo oxidation of codeine to morphine by brain membranes (Zhou et al, 2013). The formation of dopamine and serotonin by CYP2D in brain has also been assessed in vivo, through in vivo brain microdialysis measurement of neurotransmitter levels after exogenous administration of CYP2D-substrate precursors. Rats administered tyramine and 5-methyltryptamine had higher brain levels of dopamine and serotonin, respectively,
suggesting in vivo brain CYP2D formation of neurotransmitters (Bromek et al, 2011; Haduch et al, 2015).

1.4.4.2 Brain CYP2D regulation

While hepatic CYP2D levels are primarily regulated by genetics and are considered constitutively expressed, brain CYP2D activity can be altered by both genetics and by exposure to xenobiotic inducers. Single doses of the solvent toluene and the antipsychotic clozapine given to rats increased immunohistochemical staining of CYP2D4 in the olfactory bulbs, cerebellum, ventral tegmental area, and substantia nigra (Hedlund et al, 1996). There were no changes in brain CYP2D mRNA transcript levels suggesting the increase in CYP2D protein levels was due to a non-transcriptional mechanism (Hedlund et al, 1996). Additionally, while toluene produced a robust increase in liver CYP2D4 protein and mRNA levels, clozapine did not, indicating a unique brain-specific induction of CYP2D (Hedlund et al, 1996). Antipsychotics and antidepressants influence brain and liver CYP2D expression in drug-specific manners. For example, treatment for two-weeks with the antipsychotic thioridazine, shown to inhibit rat liver CYP2D, increased rat CYP2D protein levels in the substantia nigra and cerebellum (Daniel et al, 2005; Haduch et al, 2011). Single injections of the antidepressants fluoxetine and imipramine decreased brain and liver CYP2D expression (Haduch et al, 2011). However, after chronic (two-week) treatment, fluoxetine paradoxically produced region-specific increases (cerebellum) and decreases (striatum and nucleus accumbens) in brain CYP2D levels, while imipramine did not alter brain CYP2D (Haduch et al, 2011).

CYP2D6 levels are higher in the post-mortem brains of smokers and alcoholics, compared to non-smokers and non-alcoholics, while liver CYP2D6 levels do not differ (Mann et al, 2008; Miksys et al, 2002; Miksys and Tyndale, 2004). Consistent with this, short term nicotine pretreatment (seven days, subcutaneously), compared to saline, increased rat brain CYP2D enzyme levels in the cerebellum, hippocampus, and striatum (Figure 9) (Yue et al, 2008). The change in brain CYP2D expression was maximal at 8 hours after the last nicotine administration, a time where there was no detectable nicotine in plasma, and returned to baseline at 12 hr (Micu et al, 2003; Yue et al, 2008). There was no change in liver CYP2D expression, suggesting the induction occurred in brain alone (Yue et al, 2008). Furthermore, while brain CYP2D protein was increased there was no change in brain CYP2D mRNA levels, indicating the induction occurred by a non-transcriptional
mechanism (Yue et al., 2008). The precise mechanism behind the induction of brain CYP2D by nicotine is currently unknown, but may involve increased protein stability or decreased protein degradation; fourteen-day nicotine treatment downregulates enzymes involved in the ubiquitin-proteasome pathway of protein degradation in rat hypothalamus, suggesting nicotine may regulate this degradation pathway for CYP2D (Kane et al., 2004).

**Figure 9:** Fold change in brain CYP2D protein in human smokers, compared to non-smokers, and 7-day short-term nicotine pretreated rats, compared to saline pretreated. *p<0.05, **p<0.01, and ***p<0.001 according to respective analyses. (A) FC, frontal cortex; TC, temporal cortex; CG, cingulate gyrus; OC, occipital cortex; HC, hippocampus; EC, entorhinal cortex; CD, caudate nucleus; PT, putamen; NA, nucleus accumbens; GP, globus pallidus; SN, substantia nigra; Cv, cerebellar vermis; Ch, cerebellar hemisphere. (B) BS, brain stem; FC, frontal cortex; HC, hippocampus; TH, thalamus; ST, striatum; CB, cerebellum. Adapted from Mann et al. 2008 and Yue et al. 2008.

Brain CYP2D may also undergo endogenous regulation. Liver CYP2D6 levels in humans increase immediately after birth to adult levels and remain constant with age (Treluyer et al., 1991); however, CYP2D6 enzyme levels assessed in frontal cortex increased with age (tested from fetal to 80 years of age), an effect that may occur in other brain regions as well (Mann et al., 2012). Brain CYP2D expression and mRNA levels in female rats were increased after short term (seven days, subcutaneous) estrogen and testosterone treatment, and decreased after progesterone (Baum...
and Strobel, 1997; Bergh and Strobel, 1996). In contrast, short term testosterone treatment decreased brain CYP2D expression and mRNA levels in orchiectomized male rats, with no effect on liver CYP2D (Li et al, 2015a). In culture, testosterone increased levels of brain-specific microRNA (miR-101 and miR-128-2) in human brain-derived cell lines (SH-SY5Y and U251), while overexpression of microRNA decreased CYP2D mRNA levels in these cells (Li et al, 2015a). Sex hormone induced changes in these microRNA (short non-coding RNA segments involved in silencing gene expression), may be one mechanism for the sex-specific regulation of human brain CYP2D6.

1.4.4.3 Functional impact of variation in brain CYP2D
1.4.4.3.1 Neurotoxicity
A large number of centrally acting neurotoxins (e.g. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP], tetrahydroisoquinoline, paraquat, and harmaline) are metabolized by CYP2D (Buratti et al, 2003; Miksys and Tyndale, 2006). Brain CYP2D inactivation of neurotoxins to less neurotoxic metabolites locally, in regions affected by neurotoxin action, may contribute to individual susceptibility to neurotoxicity. Parkinson’s disease is a neurodegenerative disorder with both familial and sporadic etiology; environmental exposure to neurotoxins likely contributes to Parkinson’s disease (Elbaz et al, 2004; Vance et al, 2010). There is a well-established association between genetic variation in CYP2D6 and susceptibility to Parkinson’s disease. In a case-control study, CYP2D6 PMs were at a greater overall risk for developing Parkinson’s disease (Odds Ratio = 1.41, 95% Confidence Interval [CI] = 0.81-2.44) which was increased with exposure to pesticides (Odds Ratio = 1.55, 95% CI = 1.00-2.55) (Elbaz et al, 2004). Moreover, Parkinson’s disease patients were found to have 40% lower CYP2D6 levels in the frontal cortex and cerebellum than age-matched genotype-matched controls, suggesting that low brain CYP2D6 levels may be a risk factor for Parkinson’s disease (Mann et al, 2012). The neurotoxin MPTP and its toxic metabolite MPP+ are metabolized to inactive metabolites by CYP2D6; both are used in experimental animal models of Parkinson’s disease (Mann and Tyndale, 2010). Overexpressing CYP2D6 in rat adrenal medulla cells (PC12) protected against MPP+ neurotoxicity, while inhibiting CYP2D6 (with quinidine, propranolol, metoprolol, or timolol) in human neuroblastoma cells (SH-SY5Y) increased MPTP and MPP+ neurotoxicity (Mann and Tyndale, 2010; Matoh et al, 2003). Interindividual variation in the activity or expression of CYP2D6, specifically in the
brain, may contribute to differences in neurotoxin metabolic inactivation and subsequent neurotoxicity, like that seen in Parkinson’s disease.

While CYP2D6 phenotypes have been used as predictors of neurotoxicity risk, variable brain CYP2D activity through environmental exposure to xenobiotic inducers may be a novel source of variation in individual susceptibility to neurotoxicity. Smokers are at a lower risk for Parkinson’s disease, and nicotine has been found to be neuroprotective in several neurotoxin-induced animal models of Parkinson’s disease (Alves et al., 2004; Quik et al., 2009; Quik et al., 2006). Short-term nicotine treatment (two weeks via minipump) in mice protected against MPTP-induced damage in the neostriatum and substantia nigra, two regions largely affected in Parkinson’s disease (Janson et al., 1992). Mice exposed to cigarette smoke (for one week) were also protected against MPTP-induced neuronal degeneration in the substantia nigra (Parain et al., 2003). Increased brain CYP2D activity in animals given nicotine, consistent with increased brain CYP2D6 levels in smokers, may contribute to protection against Parkinson’s disease by increased CYP2D metabolic inactivation of neurotoxins (Miksys and Tyndale, 2006). Brain CYP2D levels are higher in alcoholics, compared to non-alcoholics, and are increased in rats given a single dose of intraperitoneal ethanol (Miksys et al., 2002; Warner and Gustafsson, 1994). Case-control studies have found that alcohol consumption is weakly associated with protection against Parkinson’s disease; similar to with smoking or nicotine exposure in rats, increased brain CYP2D levels observed in alcoholics may play a neuroprotective role against Parkinson’s disease (Bettiol et al., 2015).

Evidence of a functional role for brain CYP2D metabolism in drug-induced neurotoxicity has recently been demonstrated in vivo. Haloperidol is an antipsychotic drug and CYP2D substrate, associated with extra-pyramidal side-effects; catalepsy, measured as the amount of time a rodent spends immobilized with its paws on a raised surface, is used as a model of acute haloperidol induced extra-pyramidal symptoms (Castagne et al., 2009; Creed and Nobrega, 2013). Inhibiting CYP2D specifically in rat brain, by intracerebroventricular pretreatment with the mechanism-based inhibitor propranolol, decreased catalepsy; inducing CYP2D specifically in rat brain with short-term nicotine pretreatment increased catalepsy, an effect that was blocked by inhibiting brain CYP2D (Figure 10) (Miksys et al., 2017). This suggested that brain CYP2D metabolized haloperidol to a neurotoxic metabolite, responsible for producing catalepsy, and that reducing
CYP2D activity in the brain via direct administration of an inhibitor was neuroprotective for acute haloperidol induced toxicity. In support of this, haloperidol catalepsy correlated with brain, but not liver, CYP2D activity (Miksys et al., 2017). There is some evidence that antipsychotic side-effects are more prevalent in smokers (Wagner et al., 1988); increased brain CYP2D levels in smokers may contribute to a greater risk for these acute extrapyramidal side-effects.

Figure 10: Modifying rat brain CYP2D activity alters catalepsy after acute haloperidol administration. Compared to vehicle (V), inhibiting brain CYP2D (with propranolol, PL) decreased, inducing brain CYP2D (with nicotine, NIC) increased, and inhibiting the induced brain CYP2D (NIC + PL) did not alter, catalepsy after acute haloperidol (A). Catalepsy correlated with brain (B) but not liver (C) *ex vivo* CYP2D activity. *p<0.01 and **p<0.001 vs. vehicle and #p<0.01 and ##p<0.001 vs. inhibitor and inducer, using 2-way ANOVA. Relationships between data sets were assessed by Spearman’s correlation coefficients. Adapted from Miksys et al. 2017.

While acute haloperidol induces catalepsy, chronic haloperidol exposure produces vacuous chewing movements (VCM) in rats, a model of the chronic side effect tardive dyskinesia (Castagne et al., 2009; Creed and Nobrega, 2013). Inhibiting brain specific CYP2D (by intracerebroventricular propranolol pretreatment) during chronic haloperidol administration increased vacuous chewing movements (Figure 11) (Miksys et al., 2017). This suggests that haloperidol itself, or an alternative non-CYP2D metabolite, may be responsible for the chronic side effect of tardive dyskinesia and this may be exacerbated by inhibiting brain CYP2D; VCMs correlated negatively with brain, but not liver, CYP2D activity (Miksys et al., 2017). Elucidating the role of brain CYP2D in haloperidol metabolism introduces important considerations for the underlying neurochemistry of acute (catalepsy, likely mediated by a CYP2D-formed metabolite) and chronic (VCMs, likely mediated by the parent drug haloperidol or a non-CYP2D-mediated
metabolite) haloperidol-induced side effects and adds to the understanding of brain CYP2D in neurotoxicity.

**Figure 11:** Modifying rat brain CYP2D activity alters vacuous chewing movements after chronic haloperidol administration. Compared to vehicle (V), inhibiting brain CYP2D (with propranolol, PL) increased VCMs during weeks 12–16 of chronic haloperidol (A). VCMs negatively correlated with brain (B) but not liver (C) CYP2D activity. *p<0.01 using independent samples t-test. Relationships between data sets were assessed by Pearson correlation coefficients. Adapted from Miksys et al. 2017.

1.4.4.3.2 Endogenous neurochemicals

Speculation about a role for brain CYP2D in the metabolism of endogenous neurochemicals *in vivo* was initially derived from associations between CYP2D6 phenotypes and personality (Penas-Lledo and Llerena, 2014). Resting brain perfusion, measured through magnetic resonance imaging, is an indicator of cerebral activity and may be associated with personality traits; CYP2D6 PMs, compared to EMs, exhibit lower resting brain perfusion in the thalamus and hippocampus as well as more anxious personality traits (Abler *et al.*, 2008; Gonzalez *et al.*, 2008; Kirchheiner *et al.*, 2011). *CYP2D6* genotype, ranked by activity score, was associated with cortical brain activation in cognitive tests of working memory and emotional face matching, further suggesting a physiological role for brain CYP2D6 in CNS function (Stingl *et al.*, 2012). *In vitro* and *in vivo* studies in experimental animals have shown a potential role for brain CYP2D in neurotransmitter formation. Compared to wild type CYP2D6 enzyme expressed in recombinant bacteria, reduced function allelic *CYP2D6* variants had lower hydroxylation of progesterone and tyramine (Niwa *et al.*, 2004). Rat brain membranes formed dopamine from tyramine *in vitro*, a reaction reduced by the CYP2D inhibitor quinine and anti-CYP2D4 antibodies (Bromek *et al.*, 2010). Similarly, rat
brain membranes, as well as cDNA-expressed rat CYP2D and human CYP2D6, formed serotonin from exogenous 5-methyltryptamine, a reaction reduced by CYP2D inhibitors quinine and fluoxetine (Haduch et al., 2013). Rat brain CYP2D formation of dopamine and serotonin in vivo has been assessed using brain microdialysis (Bromek et al., 2011; Haduch et al., 2015). After inhibiting the primary pathways of dopamine and serotonin production, dopamine and serotonin levels were increased in vivo after exogenous administration of CYP2D-substrate precursors (tyramine for dopamine, and 5-methyltryptamine and melatonin for serotonin) (Bromek et al., 2011; Haduch et al., 2015; Haduch et al., 2016). Humanized transgenic mice (with human CYP2D6 protein expressed in the liver and brain along with normal mice CYP2D protein) have higher basal brain serotonin levels relative to wild type mice (Cheng et al., 2013). The absolute functional role of brain CYP2D in neurotransmitter homeostasis in the normal physiologically functioning CNS is currently unknown.

1.4.4.4 Brain CYP2D metabolism of opioids
There is a well-established influence of CYP2D6 phenotype on opioid metabolism and response (discussed in introduction section 1.3). This is due, largely, to CYP2D6-mediated metabolites of opioids generally displaying a much greater potency at MORs compared to the parent compounds (e.g. CYP2D-formed metabolites morphine and oxymorphone have a 3000-fold and 44-fold greater MOR affinity than the parent compounds codeine and oxycodone, respectively) (Lalovic et al., 2006; Pert and Snyder, 1973). In general, CYP2D6 metabolism occurs in the liver, after which opioid parent and/or metabolite compounds can cross the BBB to interact with MORs in the CNS and elicit analgesia. However, the expression of CYP2D6 enzyme in human brain, and demonstration of brain CYP2D activity in vivo using experimental animal models, suggests that opioids may be metabolized locally at their target site of action in the CNS.

Initial evidence of potential brain CYP2D metabolism of opioids in situ was derived from peripheral and central drug levels of codeine and its CYP2D metabolite morphine in rats (briefly discussed in 1.4.4.1). Rats were given an intraperitoneal dose of codeine or morphine that produced similar plasma morphine concentrations at 30 min post-injection, but with brain morphine levels detectable only in animals given codeine (Chen et al., 1990). This suggested that the central morphine levels at this time point after injection of codeine may not be due to peripheral morphine
levels crossing the BBB, but instead to codeine metabolism within the brain (Chen et al., 1990). Morphine is much less permeable across the BBB than codeine; using radiolabelled compounds given intra-arterially, measured as a percentage compared to freely diffusible tritiated water, rat brain morphine uptake after 15 seconds was negligible, while codeine uptake was 24% (Bouw et al., 2000; Oldendorf et al., 1972). Similarly, using pharmacokinetic-pharmacodynamic modelling of the BBB transport of oxycodone and its CYP2D metabolite oxymorphone in rats, oxymorphone brain-to-plasma partitioning was 1.6-fold lower than oxycodone (Bostrom et al., 2006; Sadiq et al., 2013). Comparing CNS influx and efflux clearances, the net uptake of oxymorphone into the CNS was 16.2-fold less than oxycodone (Bostrom et al., 2006; Sadiq et al., 2013). Oxycodone, like codeine, appears to cross the BBB much more readily than oxymorphone, like morphine, and therefore may also be impacted by brain CYP2D metabolism.

Since parent opioid compounds cross the BBB more rapidly compared to their peripherally formed CYP2D metabolites, central CYP2D metabolism of opioids may contribute to the pharmacologic effect of opioids. Suggestive of this, selectively inhibiting brain CYP2D by pretreatment with the mechanism-based inhibitor propranolol, and separately with the competitive inhibitor propafenone, decreased codeine analgesia over the first 60 min after subcutaneous codeine administration (Zhou et al., 2013). The two brain CYP2D inhibitor pretreatments decreased codeine metabolism in brain, but not liver (measured by morphine levels and morphine-to-codeine ratios), and decreased ex vivo codeine metabolism in brain membranes, but not liver microsomes (Zhou et al., 2013). Morphine analgesia was assessed as a control for the effect of brain CYP2D inhibitor and/or inducer pretreatments on opioid-mediated analgesia; inhibiting brain CYP2D did not alter analgesia across 120 min after subcutaneous morphine administration (Zhou et al., 2013). This proof of concept study showed, for the first time, the contribution of brain CYP2D to opioid metabolism and analgesia in vivo. However, it is unknown whether brain CYP2D can play a functional role in opioid analgesia across a range of brain CYP2D activity (increased and decreased), routes of opioid administration mirroring human use, multiple functional outcomes of opioid response, and multiple opioid compounds with differing relative contributions of CYP2D-formed metabolites to opioid analgesia. Investigation into the potential interindividual variability in brain opioid metabolism and response may meaningfully contribute to knowledge of variation in individual response to opioids clinically.
1.5 – Statement of Research Hypotheses

In Chapter 1, “Nicotine increases codeine analgesia through the induction of brain CYP2D and central activation of codeine to morphine”, we hypothesized that initial brain morphine levels from codeine administration were due to brain CYP2D metabolism. We further hypothesized that inducing brain CYP2D with seven days of nicotine pretreatment would increase brain morphine levels, and analgesia, despite significant first pass metabolism from intraperitoneal codeine administration, and that the impact of inducing brain CYP2D could be reversed by inhibiting brain CYP2D. Due to our pretreatment paradigms being utilized to alter brain CYP2D specifically, we hypothesized that inducing brain CYP2D would not alter plasma morphine levels from codeine. We also hypothesized that inducing brain CYP2D with nicotine would not alter baseline nociception or morphine-induced analgesia (not a CYP2D substrate).

In Chapter 2, “Inducing rat brain CYP2D with nicotine increases the rate of codeine tolerance; predicting the rate of tolerance from acute analgesic response”, we hypothesized that codeine tolerance would develop as a function of central morphine formation by brain CYP2D; inducing rat brain, but not hepatic, CYP2D with seven days of nicotine pretreatment would result in a greater proportional drop in codeine analgesia (greater rate of tolerance) during repeated daily oral codeine exposure. We further hypothesized that inducing brain CYP2D with nicotine would not alter the rate of morphine tolerance (not a CYP2D substrate) during repeated daily oral morphine exposure. Since we hypothesized a proportional drop in codeine analgesia across repeated administration, we speculated that the rate of tolerance may be a function of acute analgesic response, with nicotine pretreatment altering the rate of codeine tolerance simply by augmenting acute codeine analgesia by inducing the brain CYP2D activation of codeine.

In Chapter 3, “Rat brain CYP2D activity alters in vivo central oxycodone metabolism, levels, and resulting analgesia”, we hypothesized that oxycodone, rather than the more potent opioid-receptor activating CYP2D metabolite oxymorphone, was responsible for oxycodone analgesia, and that increasing and decreasing brain CYP2D activity would decrease and increase, respectively, central oxycodone levels, thereby oxycodone analgesia. We further hypothesized that pretreatments used to modify brain CYP2D would not affect oxymorphone analgesia (not a CYP2D substrate), baseline nociception, or peripheral oxycodone metabolism.
2. Chapter 1: Nicotine increases codeine analgesia through the induction of brain CYP2D and central activation of codeine to morphine

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Dr. Rachel F. Tyndale and Douglas M. McMillan designed the overall study. DMM conducted all animal experiments, performed the data analysis, prepared plasma and brain samples for HPLC detection, and wrote the manuscript. Fariba Baghai Wadji assisted in animal cannulation surgeries. Dr. Bin Zhao performed all HPLC procedures for codeine and morphine quantification. RFT guided the manuscript writing and edited the manuscript.
2.1 Abstract

CYP2D metabolically activates codeine to morphine, which is required for codeine analgesia. Permeability across the blood–brain barrier, and active efflux, suggests that initial morphine in the brain after codeine is due to brain CYP2D metabolism. Human CYP2D is higher in the brains, but not in the livers, of smokers and 7-day nicotine treatment induces rat brain, but not hepatic, CYP2D. The role of nicotine-induced rat brain CYP2D in the central metabolic activation of peripherally administered codeine and resulting analgesia was investigated. Rats received 7-day nicotine (1 mg/kg subcutaneously) and/or a single propranolol (CYP2D mechanism-based inhibitor; 20 μg intracerebroventricularly) pretreatment, and then were tested for analgesia and drug levels following codeine (20 mg/kg intraperitoneally) or morphine (3.5 mg/kg intraperitoneally), matched for peak analgesia. Nicotine increased codeine analgesia (1.59X AUC\textsubscript{0–30 min} vs vehicle; \(p<0.001\)), while propranolol decreased analgesia (0.56X; \(p<0.05\)); co-pretreatment was similar to vehicle controls (1.23X; \(p>0.1\)). Nicotine increased, while propranolol decreased, brain, but not plasma, morphine levels, and analgesia correlated with brain (\(p<0.02\)), but not plasma (\(p>0.4\)), morphine levels after codeine. Pretreatments did not alter baseline or morphine analgesia. Here we show that brain CYP2D alters drug response despite the presence of substantial first-pass metabolism of codeine and further that nicotine induction of brain CYP2D increases codeine response \textit{in vivo}. Thus, variation in brain CYP2D activity, due to genetics or environment, may contribute to individual differences in response to centrally acting substrates. Exposure to nicotine may increase central drug metabolism, not detected peripherally, contributing to altered drug efficacy, onset time, and/or abuse liability.
2.2 Introduction
The majority of cytochrome P450 (CYP)-mediated drug metabolism occurs in the liver; however, resulting plasma drug and/or metabolite levels do not always correlate with therapeutic effect (Ding and Kaminsky, 2003). Extra-hepatic CYP activity, specifically in the brain, may influence target-tissue drug concentration and effect (Krishna and Klotz, 1994; Michels and Marzuk, 1993). Experimentally, this has been explored, for example, by inhibiting rat brain CYP2B, which decreased the inactivation of the sedative-hypnotic propofol, resulting in a prolonged propofol effect and decreased the activation of the organophosphate chlorpyrifos reducing neurotoxicity (Khokhar and Tyndale, 2011, 2012).

CYP2D6 (herein referred to as CYP2D for all species) metabolizes a large proportion of drugs acting in the central nervous system (CNS) (e.g., amphetamine, haloperidol, and antidepressants), as well as endogenous neurochemicals (Zanger et al, 2004). The human CYP2D gene is genetically polymorphic, resulting in a wide range of metabolism phenotypes (Gaedigk et al, 2008), while brain CYP2D levels are altered by common compounds and increase with age (Mann et al, 2012; Miksys and Tyndale, 2004; Warner and Gustafsson, 1994; Yue et al, 2008). Rat and monkey brain, but not liver, CYP2D is induced by nicotine pretreatment, consistent with higher levels of human brain, but not liver, CYP2D in smokers (Miksys and Tyndale, 2004; Miller et al, 2014; Yue et al, 2008). Thus, the combination of genetics, environmental inducers, and age contribute to a wide range of brain CYP2D activity, which may alter the metabolism and efficacy of CNS-acting substrate drugs.

CYP2D metabolizes codeine to its primary analgesic metabolite morphine, a step required for codeine analgesia (Adler et al, 1955; Chen et al, 1991b; Pert and Snyder, 1973; Sindrup et al, 1992; Sindrup et al, 1990). This occurs in the liver, after which morphine crosses the blood–brain barrier (BBB) to interact with opioid receptors in the CNS and elicit analgesia. However, morphine is less permeable across the BBB than codeine and is actively effluxed (Bouw et al, 2000; Oldendorf et al, 1972). Coupled with this, brain concentrations of morphine after codeine administration do not appear to be solely attributable to transfer of morphine across the BBB (Chen et al, 1990; Zhou et al, 2013). Thus, following codeine administration, the initial morphine present
in the brain, and initial analgesia\(^1\), may be due to brain CYP2D-mediated metabolism, rather than hepatic metabolism.

Our objectives were to model the role of human brain CYP2D in response to centrally acting drugs, through investigating rat brain CYP2D metabolic activation of codeine to morphine, in the presence of hepatic first-pass metabolism, in a rat model of codeine analgesia. Rat brain CYP2D has the ability to metabolize human CYP2D probe drugs and can be inhibited and induced \emph{in vivo}, independently of hepatic CYP2D, allowing the modelling of a wide range of CYP2D activity within the brain, as postulated to exist in a heterogeneous population (Hiroi \emph{et al}, 2002; Zhou \emph{et al}, 2013). Whether the nicotine induction of brain CYP2D is sufficient to alter drug response \emph{in vivo}, using a peripheral route of drug administration with substantial first-pass metabolism by the liver, is unknown. This study aims to demonstrate that variable brain CYP2D alters substrate drug metabolism and efficacy and provides further understanding to the functional role of CYP enzymes in the brain.

2.3 Materials and Methods

\textbf{Animals}

Adult male Wistar rats (250–500 g; Charles River, St-Constant, QC, Canada) were housed in pairs or triplets with \textit{ad libitum} access to food and water. Rats were maintained under a 12-h artificial light/dark cycle with experimentation occurring during the light cycle. Stress was reduced through acclimation to experimenters and apparatuses. All procedures were approved by the Animal Care Committee at the University of Toronto.

\textbf{Drug Treatment}

Codeine phosphate and morphine sulfate (PCCA, London, Canada) were dissolved in saline (0.9% NaCl; pH 7) and distilled water, respectively, and injected intraperitoneally (i.p.). The doses of codeine (20 mg/kg) and morphine (3.5 mg/kg) were chosen to match peak analgesia after testing multiple doses of codeine (20, 25 and 30 mg/kg) and morphine (0.5, 1, 2, and 3.5 mg/kg). Propranolol hydrochloride (Sigma-Aldrich, Oakville, Canada) was dissolved in artificial

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\(^1\)“Initial analgesia”, as described throughout this chapter, is equivalent to “acute analgesia” as referred to in chapters 1 and 3
cerebrospinal fluid to deliver 20 μg base in 4 μl via intracerebroventricular (i.c.v.) injection 24 h prior to codeine or morphine administration. Propranolol is a CYP2D mechanism-based inhibitor, metabolized by CYP2D; the metabolite covalently binds and inactivates CYP2D (Narimatsu et al, 2001). Propafenone hydrochloride (Sigma-Aldrich), a CYP2D competitive inhibitor (Xu et al, 1995), was dissolved in a 20% (w/v) solution of 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) in water to deliver 40 μg base in 4 μl i.c.v., 5 min prior to codeine or morphine administration. Two mechanismically and structurally distinct CYP2D inhibitors were used to reduce the possibility of off-target effects other than inhibition of codeine metabolism. Nicotine bitartrate (Sigma-Aldrich) was dissolved in saline and given subcutaneously (s.c.) at 1 mg base/kg daily for 7 days, which results in elevated rat brain, but not liver, CYP2D levels as previously described (Yue et al, 2008).

**Cannulation Surgeries for Multiple i.c.v. Injections**

Rats utilized for brain CYP2D-inhibition experiments were surgically implanted with i.c.v. cannulas into their right lateral ventricle (Zhou et al, 2013). Screw anchors (BASi, West Lafayette, USA) were implanted into the skull to serve as an anchor to the DuraLay inlay pattern resin (Reliance Dental Mfg. Co., Worth, USA) used as an adhesive for the implanted cannula.

**Nociceptive Testing of Codeine and Morphine Analgesia**

Analgesia was measured by the tail-flick reflex test through the use of a tail-flick meter (Columbus Instruments, Columbus, OH, USA) (D'Amour and Smith, 1941). A thermal stimulus is applied to the rat's tail, −5 cm from the distal end, and the time of onset of stimulus to a vigorous tail flick is recorded as the tail-flick latency (TFL). Prolongation of the TFL is an indication of both spinally and supra-spinally mediated analgesia (Le Bars et al, 2001). The thermal strength of the tail-flick meter was adjusted to obtain baseline TFLs around 3 s; a cutoff of 10 s was used to avoid damage. TFLs were measured twice in each rat 24 h prior to experimentation, and the mean was used as the individual rat's baseline TFL. Analgesia was expressed as a percentage of the maximal possible effect (%MPE) (Dewey and Harris, 1971).

\[
\%\text{MPE} = \left( \frac{\text{postinjection latency} - \text{baseline latency}}{\text{maximum cutoff} - \text{baseline latency}} \right) \times 100\%
\]
Analgesia Experimental Design

For pretreatment experiments, codeine or morphine was given 24 h after i.c.v. pretreatment injection (propranolol or vehicle) and/or 8 h after the last s.c. pretreatment injection (nicotine or saline). After opioid administration, analgesia was assessed by TFL every 5 min for 30 min and then every 10 min for the next 30 min. Within-animal designs were used where possible in which, after a 1-week washout period, rats were crossed over to a comparator pretreatment group and retested or killed for pharmacokinetic analysis.

Plasma and Brain Sample Preparation

For analysis of codeine and morphine from the plasma and brain, rats were pretreated, then administered codeine, tested for TFL at 15 min postinjection, and killed by decapitation. Trunk blood was centrifuged at 5000 g for 10 min. Half-brains were homogenized in 1:3 (w/v) 0.01 N HCl and centrifuged at 5000 g for 10 min. Nicotine plasma samples were obtained on the seventh day of nicotine pretreatment via saphenous-vein blood draws.

HPLC Measurement of Plasma and Brain Drug/Metabolite Levels

Plasma and brain homogenate samples were analyzed as previously described (Zhou et al., 2013). In brief, 100 μl of solid phase extract was analyzed by HPLC with ultraviolet detection at 214 nm (Agilent 1200 Separation Module, Palo Alto, CA, USA). Morphine, codeine, and internal standard (1 μg 2-benzoxazolinone) were separated on an Agilent ZORBAX SB-C18 Column (250 mm × 4.6 mm I.D.; particle size, 5 μm), with the retention times of 4.1, 9.0 and 16.7 min, respectively. The mobile phase used was methanol: phosphate buffer (29.3: 70.7 (v/v); pH 5.8) with a flow rate of 1 ml/min. The limits of quantification were 5 and 10 ng/ml (5 and 50 ng/g for brain tissue) for morphine and codeine, with an extraction efficiency of 76.9 and 90.1%, respectively. Nicotine and cotinine were quantified by HPLC (Siu et al., 2006).

Statistical Analyses

Data were analyzed through one-way or two-way repeated-measures ANOVA with Bonferroni corrections or unpaired (between animal) and paired (within animal) one-tailed t-tests, as indicated.
2.4 Results

**Analgesic Profiles and Morphine Concentrations Suggest Involvement of Brain CYP2D in Codeine Metabolism and Initial Codeine Analgesia**

Codeine (20 mg/kg) resulted in a peak of analgesia around 15 min ($n=22$; Figure 12A) with morphine metabolite levels detectable in both plasma and brain ($n=5$; Figure 12B and 12C). This codeine dose used throughout provided analgesia below maximal %MPE, allowing for measurement of both increases and decreases in analgesia. Morphine (3.5 mg/kg) matched the peak analgesia level obtained from codeine, however, later at 30 min ($n=8$; Figure 12A). At 15 min, the morphine dose produced similar plasma morphine levels to those from codeine ($p>0.3$; $n=10$; Figure 12B), but lower brain morphine levels ($p<0.02$; Figure 12C), consistent with lower analgesia at this time point. This suggests that the early analgesia peak at 15 min following codeine, along with the high levels of brain morphine present, was due to morphine formed by central (brain) metabolism. The delayed peak in analgesia after morphine suggests that peripheral morphine, as compared with the more lipophilic codeine, crosses the BBB more slowly, therefore eliciting analgesia at a later time.
Figure 12: The analgesic time course following codeine and morphine suggests peripheral morphine levels do not predict central morphine levels or analgesia. Codeine (20 mg/kg) produced an analgesic peak around 15 min postinjection ($T_{\text{MAX}}=13+1.8 \text{ min, mean+SEM}; n=22$; a). A morphine dose (3.5 mg/kg), chosen to match peak analgesia from codeine, resulted in an equivalent albeit later analgesia peak around 30 min postinjection ($T_{\text{MAX}}=27+4.9 \text{ min}; n=8$). At 15 min postinjection, the time of the codeine analgesia peak, the morphine dose ($n=10$) produced similar plasma morphine levels to the codeine dose ($n=5$; b), and lower brain morphine levels (c) consistent with the lower analgesia. A between-animal study design was used; *$p<0.05$ compared with codeine (20 mg/kg) using an unpaired $t$-test.

Pretreatment with Two Distinct CYP2D Inhibitors (i.c.v.) Decreased the First 30 min of Codeine-Induced Analgesia but Did Not Affect Morphine-Induced Analgesia

With a focus on the time frame (0–30 min postinjection) hypothesized to be associated with brain metabolism of codeine to morphine and the resulting peak in codeine-induced analgesia, the effect of inhibiting brain CYP2D was assessed using two structurally and mechanistically distinct CYP2D inhibitors. Compared with vehicle pretreatment, 20 μg propranolol (mechanism-based inhibitor) pretreatment resulted in significantly lower %MPE at numerous time points ($n=16$/group; Figure 13A) after codeine (20 mg/kg) administration, producing a significant reduction of the analgesic AUC$_{0-30 \text{ min}}$ ($p<0.01$; Figure 13B). Likewise, 40 μg propafenone
(competitive inhibitor) pretreatment resulted in significantly lower %MPE ($n=6$/group; Figure 13D, and analgesic $AUC_{0-30\text{ min}}$ after codeine administration ($p<0.05$; Figure 13E).}

![Graphs showing analgesia and AUC comparisons](image)

**Figure 13:** Inhibiting brain CYP2D reduced the first 30 min of codeine-induced analgesia but had no effect on morphine-induced analgesia. Compared with vehicle pretreatment, propranolol pretreatment (darker bar) resulted in significantly lower analgesia (%MPE; a) and area under the analgesia–time curve ($AUC$; b) for 0–30 min after codeine administration ($n=16$/group). Propranolol pretreatment (darker bars) did not result in a difference in analgesic $AUC_{0-30\text{ min}}$ or $AUC_{30-60\text{ min}}$ (c) after morphine administration ($n=5$/group; hatched bars). Compared with vehicle pretreatment, propafenone pretreatment (darker bar) resulted in significantly lower %MPE (d) and analgesic $AUC_{0-30\text{ min}}$ (e) after codeine administration ($n=6$/group). Propafenone pretreatment (darker bars) did not result in a difference in analgesic $AUC_{0-30\text{ min}}$ or $AUC_{30-60\text{ min}}$ (f) after morphine administration ($n=5$/group; hatched bars). Error bars indicate SEM. A within-animal study design was used; for analgesia–time curves, *$p<0.05$, **$p<0.01$, and ***$p<0.001$ using repeated-measures ANOVA with Bonferroni *post hoc*. For AUCs, *$p<0.05$, **$p<0.01$ using paired $t$-tests.
As morphine is the active analgesic metabolite of codeine and not further metabolized by CYP2D, its analgesia should not be altered by changes in CYP2D activity. Compared with vehicle pretreatment, neither propranolol nor propafenone pretreatment altered %MPE ($p>0.1$ at all time points) or analgesic AUC ($p>0.1$) across any time frame after morphine administration ($n=5$/group; illustrated for AUC$_{0-30\text{ min}}$ and AUC$_{30-60\text{ min}}$ in Figure 13C and 13F).

Pretreatment with CYP2D inhibitors also had no effect on baseline nociception; there was no difference between TFL prior to (3.52±0.21, mean±SEM) and 24 h after vehicle (3.64±0.16 s) or (3.72±0.20 s) propranolol pretreatment ($n=16$; $p>0.2$), or TFL prior to (3.03±0.14 s) and 5 min after vehicle (3.13±0.14 s) or (3.18±0.16 s) propafenone pretreatment ($n=6$; $p>0.2$). Together, this indicated that CYP2D inhibitor pretreatments had no effect on analgesia that was unrelated to their effects on codeine metabolism.

7-Day Pretreatment with Nicotine, to Induce Brain CYP2D, Increased the First 30 min of Codeine-Induced Analgesia but Did Not Affect Morphine-Induced Analgesia

Nicotine administration induces rat brain CYP2D without altering hepatic CYP2D; rats were administered nicotine (1 mg/kg; s.c.) or vehicle (saline, 1 ml/kg; s.c.) and tested at 8 h after the seventh and final injection (Figure 14A), when brain CYP2D is significantly induced (Yue et al, 2008). On the final day of nicotine pretreatment, plasma nicotine levels peaked within the first 30 min and were undetectable at 8 h postinjection, the time of codeine or morphine administration and analgesia testing ($n=16$; Figure 14B). Compared with vehicle pretreatment, 7 days of nicotine pretreatment resulted in significantly higher %MPE ($n=20$/group; Figure 14A) after codeine administration, producing a significantly higher AUC$_{0-30\text{ min}}$ ($p<0.03$; Figure 14B).

The effect of nicotine pretreatment on morphine analgesia (3.5 mg/kg) was also tested; if nicotine's actions on increasing codeine-induced analgesia were via induced brain CYP2D, no change in morphine-induced analgesia would be expected. Compared with vehicle pretreatment, nicotine pretreatment did not result in significantly altered %MPE ($p>0.3$ at all time points) or analgesic AUC ($p>0.3$) across any time frame after morphine administration ($n=16$/group; illustrated for AUC$_{0-30\text{ min}}$ and AUC$_{30-60\text{ min}}$ in Figure 14E).
Figure 14: Nicotine-mediated induction of brain CYP2D increased codeine analgesia and had no effect on morphine-induced analgesia. Rats were administered nicotine (1 mg/kg; s.c.) or saline vehicle (s.c.) once daily for 7 days as outlined (a). As measured on the final day of nicotine pretreatment, plasma nicotine levels peaked within the first 30 min and were undetectable by 8 h, the time of codeine or morphine administration (n=16; b). Mean plasma nicotine levels averaged over 8 h was approximately 70 ng/ml and over 24 h was approximately 25 ng/ml. Compared with vehicle pretreatment, nicotine pretreatment (darker bar) resulted in significantly higher %MPE (c) and analgesic AUC$_{0-30}$(d) after codeine administration (n=20/group). Seven days of nicotine pretreatment (darker bars) did not result in a difference in analgesic AUC$_{0-30}$ or AUC$_{30-60}$ (e) after a morphine injection (n=16/group; hatched bars). Error bars indicate SEM. A within-animal study design was used; for analgesia–time curves, **p<0.01 using repeated-measures ANOVA with Bonferroni post hoc. For AUCs, *p<0.05 using paired t-tests.

The effect of 7-day nicotine pretreatment on codeine and morphine analgesia from the tail-flick assay was also tested using the hot-plate assay (Eddy and Leimbach, 1953; Rowland et al., 1994). Compared with vehicle, nicotine pretreatment resulted in significantly higher %MPE (Supplementary, Figure 17A) and a 2.46-fold greater AUC$_{0-30}$ (p<0.01; n=8/group; Supplementary, Figure 17B) after codeine administration, with no change in %MPE (p>0.2 at all
time points) or AUC \((p>0.3)\) across any time frame after morphine administration \((n=5/group; \text{Supplementary, Figure 17C})\).

TFLs were assessed prior to daily nicotine injections and at 8 h postinjection (the time at which codeine and morphine were tested). There was no change in preinjection TFL (Day 1: 5.4±0.5 s, mean±SEM; Day 7: 5.3±0.4 s; \(p=0.39\)) or 8 h postinjection TFL (Day 1: 5.1±0.5 s; Day 7: 4.7±0.3 s; \(p=0.20\)) and no change in TFL between preinjection and 8 h postinjection \((p>0.1)\) across each of the 7 days of nicotine injections. Together, with the lack of effect on morphine analgesia, this suggests that nicotine (as tested here) did not alter nociception during codeine and morphine testing, suggesting, instead, a direct effect on codeine analgesia via induction of brain CYP2D.

**Pretreatment with a Mechanism-Based Brain CYP2D Inhibitor Blocked the Effect of Nicotine Induction of Brain CYP2D on Codeine Analgesia**

Compared with vehicle pretreatment, when animals were pretreated with 7 days of nicotine, there was a significant increase in %MPE \((n=12/group; \text{Figure 15A})\) and resulting increase in analgesic AUC_{0–30 min} after codeine \((p<0.001; \text{Figure 15B})\). Conversely, when animals were pretreated with propranolol, there was a significant decrease in %MPE and analgesic AUC_{0–30 min} after codeine administration \((p<0.05)\). Co-pretreatment of propranolol blocked the nicotine-induced increase in codeine analgesia, likely through inhibiting the induced levels of brain CYP2D, and resulted in similar codeine to the vehicle pretreatment \((p=0.8)\). There was no significant difference in baseline TFL between the four pretreatment groups \((p>0.3)\). Thus, the effect of propranolol and nicotine pretreatment together resulted in no net change in codeine-induced analgesia, suggesting that they were acting in opposing directions via the same mechanism, presumably through modification of brain CYP2D activity.
Figure 15: Nicotine induction of brain CYP2D increased the first 30 min of codeine-induced analgesia, an effect that was blocked by inhibiting brain CYP2D. Compared with vehicle pretreatment, 7 days of nicotine pretreatment or propranolol pretreatment alone resulted in significantly higher and lower, respectively, %MPE (a) and analgesic AUC$_{0-30min}$ (b) after codeine administration. Compared with vehicle pretreatment, co-pretreatment of nicotine and propranolol together resulted in a significantly different %MPE and AUC$_{0-30min}$ than nicotine or propranolol pretreatment alone and no change in %MPE or AUC$_{0-30min}$ from vehicle pretreatment ($n=12$/group). Error bars indicate SEM. A within-animal study design was used; *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared with vehicle, and #*$p<0.05$, ##*$p<0.01$, compared with the combination pretreatment, using repeated-measures ANOVA with a Bonferroni post hoc test.

Variation in Brain CYP2D Altered Brain Metabolism of Codeine Without Affecting Hepatic CYP2D

To investigate whether the alterations in codeine analgesia correlated with changes in codeine metabolism in vivo, brain and plasma codeine and morphine levels were assessed after administration of the combination of pretreatments used previously (Figure 15). There was a significant correlation between plasma and brain codeine levels ($r=0.60; p<0.001$; Figure 16A) but not between morphine levels ($r=0.14; p=0.26$; Figure 16B) at 15 min after codeine administration ($n=26$). This suggests that at 15 min after codeine administration plasma morphine levels did not predict brain morphine levels, indicating the presence of barriers to morphine permeability across the BBB. In the same analysis, analgesia at 15 min correlated with brain morphine levels ($r=0.41; p<0.02$; Figure 16C) but not with plasma morphine ($r=0.01; p=0.48$; Figure 16D) at 15 min after codeine administration ($n=26$), consistent with brain CYP2D-mediated codeine metabolism and resulting brain morphine levels, being responsible for codeine analgesia over the first 30 min after codeine administration.
Figure 16: Codeine, but not morphine, levels correlate between plasma and brain; brain morphine levels reflect codeine analgesia. Plasma and brain codeine levels correlated significantly (a) at 15 min, suggesting that codeine equilibrated quickly between the plasma and brain compartments. There was no correlation between plasma and brain morphine levels following codeine administration (b). Analgesia correlated with brain morphine levels (c) but not with plasma morphine (d) at 15 min after codeine administration (n=26). Pearson correlations were used.

Compared with vehicle pretreated rats (48.5±2.0 ng/g, mean±SEM), nicotine pretreated rats had significantly higher morphine levels in the brain (54.7±2.4 ng/g; p<0.02), propranolol pretreated trended towards lower morphine levels in the brain (44.5±2.6 ng/g; p=0.2), and rats co-pretreated with nicotine and propranolol had no difference in the brain morphine levels (51.6±2.2 ng/g; p=0.8) after codeine administration, all consistent with brain CYP2D-specific changes (n=8/group). The three active pretreatment paradigms did not alter plasma morphine levels (p>0.7, for all comparisons), suggesting that these pretreatments modified brain metabolism of codeine, without affecting hepatic metabolism.
2.5 Discussion

This is the first study to demonstrate that brain CYP2D has a role in drug response even in the presence of substantial first-pass metabolism of codeine and further that nicotine induction of brain CYP2D can increase central drug metabolism and response \textit{in vivo}. Previously, it was unknown whether the nicotine-induced levels of brain CYP2D were sufficient and possessed adequate cofactors \textit{in situ} to alter CNS-acting drug metabolism and resulting drug effect \textit{in vivo}. This is also the first study to show that this nicotine induction of brain CYP2D alters drug response and can be blocked with brain CYP2D inhibitors, suggesting the importance of the interplay of compounds that alter brain activity on centrally acting drug effect, including for orally administered drugs.

Codeine and morphine, given at doses resulting in equivalent plasma morphine levels at 15 min postinjection and peak analgesia, resulted in significantly different brain morphine levels and analgesia time lines, suggesting that hepatic metabolism of codeine to morphine was not solely responsible for the levels of brain morphine and centrally mediated analgesia after a codeine injection. Our findings suggest a significant role for variable brain metabolism in codeine analgesia; inhibiting brain CYP2D with two mechanistically distinct inhibitors decreased, while inducing brain CYP2D with nicotine increased, codeine analgesia and resulting brain morphine levels, effects that were selectively blocked by co-pretreatment. Furthermore, neither inhibiting nor inducing brain CYP2D altered baseline response, morphine, analgesia, or peripheral metabolism of codeine, suggesting that the pretreatments used to modify brain CYP2D did not have off-target effects on codeine's downstream mechanism of action.

The 7-day nicotine pretreatment paradigm resulted in average daily nicotine plasma levels of 25 ng/ml (Figure 14B), similar to those seen in human smokers (20–50 ng/ml) (Benowitz, 1999); similar to nicotine-treated animals, smoking is associated with higher human brain CYP2D (Mann \textit{et al.}, 2008; Miksys and Tyndale, 2004; Miller \textit{et al.}, 2014; Yue \textit{et al.}, 2008). Peripheral pharmacokinetics of codeine are similar in smokers and non-smokers, consistent with our current animal studies, and with a lack of effect of smoking or nicotine treatment on hepatic CYP2D (Miller, 1990). Approximately 20% of the US population continues to smoke cigarettes; smokers have a greater risk of multi-drug use and dependency (Berg \textit{et al.}, 2013). Furthermore, the use of electronic cigarettes that deliver nicotine mimicking conventional cigarettes is increasing,
particularly in adolescents (Dutra and Glantz, 2014). Opioid analgesics such as codeine, while widely accepted for the treatment of acute to chronic pain, are abused for their euphoric mood-altering properties, with the incidence of non-medicinal prescription opioid use increasing (Fischer et al, 2006). The reinforcing qualities of codeine come from its morphine metabolite, therefore for individuals with higher levels of brain CYP2D (e.g. smokers) a more rapid exposure of the brain to morphine, through faster centrally mediated metabolic activation, could speed the onset time of codeine's reinforcing effects and increase its abuse liability (Kathiramalainathan et al, 2000; Tyndale et al, 1997). Increased central opiate activation may be one mechanism increasing the risk of smoking and opioid co-dependency (Richter and Ahluwalia, 2000). In support of this, the prevalence of repeated prescription frequency, and general non-medicinal prescription opioid use, is higher in both men and women with smoking dependence (Skurtveit et al, 2010). Although alternative mechanistic explanations exist, such as receptor cross-sensitization in the enhanced rewarding and psychomotor effects of morphine in rodents following nicotine treatment, nicotine's ability to alter drug metabolism within the brain may also contribute (Vihavainen et al, 2008).

In animals pretreated with chronic nicotine, there was a trend for a faster onset of analgesia; the $T_{\text{MAX}}$ with brain CYP2D induction (14.6+1.7 min, mean+SEM) was shorter compared with vehicle pretreatment (18.8+1.4 min; $p>0.1$), brain CYP2D inhibition (17.1+1.8 min; $p>0.4$) and co-pretreatment (17.1+1.8 min; $n=12/\text{group}; p>0.5$ using a two-way ANOVA against brain CYP2D induction). A shift in codeine analgesic $T_{\text{MAX}}$, due to enhancement of brain CYP2D metabolism, could contribute to a faster onset of codeine's clinical actions but may also increase its abuse liability. Individuals exposed to compounds that affect brain CYP2D levels and/or activity could present with varied profiles of both clinical effect and abuse potential to drugs that are activated (i.e., codeine), or inactivated (i.e., amphetamine) by CYP2D (Wu et al, 1997).

The absolute magnitude of the change in morphine levels, analgesia and $T_{\text{MAX}}$ was likely blunted, due to the brain CYP2D inhibition paradigm employed, resulting from incomplete inhibition of CYP2D. A larger dose of propranolol (40 $\mu$g base in 4 $\mu$l), which reduced analgesia further, inhibited hepatic CYP2D (determined by ex vivo metabolic assays) suggesting that propranolol had crossed the BBB into the periphery and obscured the ability to focus solely on brain metabolism (Zhou et al, 2013). An active search is underway for alternative molecular-inhibition
options for future experiments. In addition, brain CYPs are highly localized and respond to inducers and inhibitors with complex patterns of regulation that are both tissue and cell-type specific (Yue et al, 2008). For this reason, the direct correlation between brain regions associated with antinociception and with CYP2D protein affected by induction and inhibition is unclear. Thus, global brain inhibition and homogenates were used as a way of capturing a more general picture, however, likely resulted in a muting of the propranolol effect. Further studies into brain regions corresponding to high opioid receptor and CYP2D protein density are warranted to elucidate the importance of region-specific brain CYP2D alterations on codeine metabolism and analgesia.

Nicotine has an important role in modulating pain transmission. Activation of the $\alpha 4\beta 2$ and $\alpha 7$ nicotinic acetylcholine receptor subtypes elicits analgesia and is purported to involve opioidergic neurons and endogenous opioid peptides; nicotine analgesia is decreased in $\mu$-opioid receptor knockout mice (Berrendero et al, 2002; Carstens et al, 2001; Damaj et al, 2000; Dhatt et al, 1995). Furthermore, nicotine dependence and withdrawal can affect both opioid- and noxious stimulus-induced analgesia involving these receptors in the nucleus accumbens (Schmidt et al, 2001). For these reasons, it was important to control for nicotine's own potential effects on nociception and opioid action. There were no detectable levels of nicotine at 8 h postinjection, the time of codeine/morphine administration (Figure 14B). Furthermore, there was no change in baseline nociception between prenicotine and 8 h postnicotine administration, consistent with the absence of nicotine or nicotine-induced nociception at this time point. Chronic nicotine administration also did not affect morphine analgesia (Figure 14E), indicating a lack of direct effect of nicotine on opioid-induced analgesia per se, including on the BBB permeability of morphine. Together, this substantially reduces the likelihood of nicotine pretreatment effects on general nociception, nicotine-mediated analgesia, or opioid-mediated analgesia in this chronic administration paradigm, suggesting instead an effect of nicotine on brain CYP2D levels.

Overall, these results suggest a role of brain CYP2D on codeine metabolism and analgesia. Following codeine administration, analgesia is initially mediated by morphine formed centrally by brain CYP2D, due to the delay in hepatically formed morphine entering the CNS. Thus lower (or higher) brain CYP2D activity and the consequent decrease (or increase) in brain morphine levels
results in lesser (or greater) analgesia during this initial 30 min after codeine injection. Over time, morphine crosses into the brain, suggesting that analgesia at this later time period is mediated by morphine formed by hepatic CYP2D crossing into the CNS (seen from the shift in analgesic peak from morphine (3.5 mg/kg) compared with codeine (20 mg/kg)). The clinical implications of our findings are that individuals with higher brain CYP2D activity, such as genetically ultrarapid CYP2D metabolizers or smokers, may experience a quicker and greater onset of analgesia (and potentially abuse liability) from codeine, whereas those with lower brain CYP2D activity, such as non-smokers or those taking CYP2D inhibitors, may experience a delayed and lower analgesia. Accordingly, our results suggest that a translational study assessing the time course and peak of codeine's analgesic effects in smokers vs non-smokers, or within subject prenicotine/postnicotine treatment, would be warranted. Furthermore, it is suggested that drug metabolism within the brain may cause interindividual differences in drug response, which are not reflected in plasma drug levels.

2.6 Acknowledgements
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2.7 Supplementary Information

**Supplementary Material: Effect of brain CYP2D induction on codeine hot-plate analgesia**

**Figure 17:** Nicotine mediated induction of brain CYP2D increased codeine analgesia, and had no effect on morphine-induced analgesia when assessed via the hot plate assay. Compared to vehicle pretreatment, nicotine pretreatment resulted in significantly higher %MPE (a) and analgesic AUC\_0-30 min (b) after codeine administration (n=8/group), with no difference in analgesia after morphine administration (c; n=5/group; hatched bars). Error bars indicate SEM. For analgesia-time curves, **p<0.01 using one-way ANOVA with Bonferroni post-hoc. For AUCs, **p<0.01 using paired t-tests.

**Materials and Methods for the Hot-Plate Assay**

A secondary technique for assessment of analgesia was employed in the hot-plate test, through the use of a hot plate analgesia meter (IITC Life Science, Woodland Hills, CA, USA). In the hot-plate test, rats are placed on a warm plate enclosed with Plexiglas walls. The latency time to either lick a hind-paw or jump was used as an endpoint measure (Eddy and Leimbach, 1953). The hot-plate temperature was set at 52.5°C, and rats were removed from the hot-plate immediately upon meeting endpoint measure, or by the cut-off period of 30 s, to avoid tissue damage. The hot-plate test employs a more complex behaviour as an endpoint, utilizing a primarily supra-spinal mechanism (Rowland et al, 1994). Similar to the tail-flick reflex assay, hot plate latencies were measured two times in each rat 24 h prior to experimentation, and the mean was used as the individual rat’s baseline latency.
2.8 Significance to Thesis

This chapter contributes to the literature by showing that altering rat brain CYP2D metabolism impacts acute, systemically administered, opioid response in vivo. This study showed that initial brain morphine levels and early analgesia after intraperitoneal codeine administration is due, in part, to brain CYP2D metabolism. Inhibiting rat brain CYP2D decreased brain morphine levels from codeine and resulting analgesia, while inducing rat brain CYP2D increased brain morphine levels from codeine and resulting analgesia. Prior to this work, it was unknown if the induction of rat brain CYP2D by short term nicotine pretreatment would be sufficient to impact drug metabolism or response in vivo. We showed that nicotine administration induced brain CYP2D activity in vivo, without affecting hepatic CYP2D activity; inducing brain CYP2D increased brain, but not plasma, morphine levels from codeine and increased ex vivo dextromethorphan (CYP2D probe substrate) metabolism in brain membranes, but not liver microsomes. We demonstrated the specificity of our induction paradigm by co-administering the brain CYP2D inhibitor propranolol; inhibiting the induced brain CYP2D reversed the effects of brain CYP2D induction on ex vivo brain CYP2D activity, and brain morphine levels and analgesia from codeine. Additional controls ensured that there was no effect of nicotine pretreatment on codeine analgesia independent of brain CYP2D induction. Nicotine pretreatment did not alter baseline nociception, assessed by tail-flick latency at 8 hr after the last nicotine pretreatment (the time of maximal brain CYP2D induction and therefore opioid administration). In addition, we found no impact of inducing or inhibiting brain CYP2D on morphine-induced analgesia (not a CYP2D-substrate) indicating our pretreatments were affecting codeine analgesia by altering brain CYP2D metabolism.

Findings from this chapter suggest that variation in brain CYP2D activity may contribute to some of the interindividual variability in opioid response. Individuals with high brain CYP2D activity (e.g. smokers, individuals taking nicotine) may respond greater to codeine, including analgesia, subjective effects, and adverse events, due to greater central metabolic activation of codeine to morphine. Comparatively, individuals with decreased brain CYP2D activity (e.g. individuals taking CYP2D inhibitors which enter the brain, genetically poor CYP2D6 metabolizers) may respond less to codeine.
3. Chapter 2: Inducing rat brain CYP2D with nicotine increases the rate of codeine tolerance; predicting the rate of tolerance from acute analgesic response

Douglas M. McMillan and Rachel F. Tyndale

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Dr. Rachel F. Tyndale and Douglas M. McMillan designed the overall study. DMM conducted all analgesia experiments and ex vivo metabolism assays, performed the data analysis, prepared plasma and brain samples for HPLC detection, and wrote the manuscript. Dr. Bin Zhao performed all HPLC procedures for codeine, morphine, dextromethorphan, and dextrorphan quantification. RFT guided the manuscript writing and edited the manuscript.
3.1 Abstract
Repeated opioid administration produces analgesic tolerance, which may lead to dose escalation. Brain CYP2D metabolizes codeine to morphine, a bioactivation step required for codeine analgesia. Higher brain, but not liver, CYP2D is found in smokers and nicotine induces rat brain, but not liver, CYP2D expression and activity. Nicotine induction of rat brain CYP2D increases acute codeine conversion to morphine, and analgesia, however the role of brain CYP2D on the effects of repeated codeine exposure and tolerance is unknown. Rats were pretreated with nicotine (brain CYP2D inducer; 1 mg/kg subcutaneously) or vehicle (saline; 1 ml/kg subcutaneously). Codeine (40–60 mg/kg oral-gavage) or morphine (20–30 mg/kg oral-gavage) was administered daily and analgesia was assessed daily using the tail-flick reflex assay. Nicotine (versus saline) pretreatment increased acute codeine analgesia (1.32-fold change in AUC$_{0-60}$ min; p < 0.05) and the rate of loss of peak analgesia (11.42%/day versus 4.20%; p < 0.006) across the first four days of codeine administration (time to negligible analgesia). Inducing brain CYP2D with nicotine did not alter acute morphine analgesia (1.03-fold; p > 0.8), or the rate of morphine tolerance (8.1%/day versus 7.6%; p > 0.9). The rate of both codeine and morphine tolerance (loss in peak analgesia from day 1 to day 4) correlated with initial analgesic response on day 1 (R = 0.97, p < 0.001). Increasing brain CYP2D altered initial analgesia and subsequent rate of tolerance. Variation in an individual’s initial response to analgesic (e.g. high initial dose, smoking) may affect the rate of tolerance, and thereby the risk for dose escalation and/or opioid dependence.
3.2 Introduction

Codeine is metabolically activated to morphine by cytochrome P450 2D6 (referred to as CYP2D for all species), a step required for codeine analgesia (Chen et al, 1991b; Sindrup et al, 1992; Sindrup et al, 1990). After codeine administration, initial morphine in the brain, and resulting analgesia are due in part to central nervous system (CNS)-expressed CYP2D-mediated formation of morphine within the brain (Chen et al, 1990; Pert and Snyder, 1973). This may be due to lower morphine entry/permeability across the blood–brain barrier, as well as morphine efflux (Bouw et al, 2000; Oldendorf et al, 1972). CYP-mediated drug metabolism primarily occurs in the liver; plasma drug and/or metabolite levels of drugs do not always correlate with response to CNS-acting drugs (Ding and Kaminsky, 2003). CYP-mediated metabolism within the brain can influence target tissue drug concentration and effect, potentially explaining some of this discrepancy, as well as contributing to inter-individual variability of drug response (Ferguson and Tyndale, 2011; Krishna and Klotz, 1994; Miksys and Tyndale, 2009, 2013).

Opioids are effective analgesics for acute (e.g. post-operative) and chronic (e.g. neuropathic) pain, however opioid drug-related emergency department visits, admissions to substance abuse treatment, and overdose deaths have increased dramatically over the past 15 years (Cai et al, 2010; Frenk et al, 2015). The United States is currently the world’s highest per-capita consumer of opioids, including those used therapeutically (e.g. codeine, oxycodone) and illicitly (e.g. heroin) (Frenk et al, 2015; Jones et al, 2015; Miech et al, 2015). One contributor to this increase in opioid use is repeated-exposure to opioids resulting in tolerance, or a loss of analgesic potency, leading to increased dose-requirements over time (Trang et al, 2015). Tolerance is a result of neuroadaptation to chronic opioid exposure and mu-opioid receptor activation, however the exact mechanisms are unknown (Williams et al, 2013). Animal studies suggest that gliosis, changes in the immune system, and central oxidative stress can play important roles, but little is known of the upstream role of variation in opioid metabolism, centrally or peripherally, in development of opioid tolerance (Fan et al, 2015; Grace et al, 2015; Johnson et al, 2014).

We have recently shown that brain CYP2D plays a role in acute codeine analgesia (McMillan and Tyndale, 2015). Inhibiting rat CYP2D selectively in the brain with a central (intracerebroventricular, i.c.v.) injection of a mechanism-based inhibitor propranolol (given 24 h
ahead of codeine) decreased brain, but not plasma, morphine levels and analgesia indicative of the inhibition of brain CYP2D’s formation of morphine from codeine (McMillan and Tyndale, 2015). We had previously shown that in vivo i.c.v. injections of propranolol inhibited brain, but not hepatic, ex vivo codeine metabolism to morphine (Zhou et al, 2013). Consistent with the action of i.c.v. propranolol being selective on inhibiting CYP2D, pretreatment had no effect on analgesia following morphine injection, which is not a substrate of CYP2D (Zhou et al, 2013). We have also shown that 7-day subcutaneous (s.c.) nicotine pretreatment induces rat brain, but not hepatic, CYP2D; brain CYP2D is elevated at 8 h after the last nicotine injection, but there is no detectable nicotine in plasma (McMillan and Tyndale, 2015; Yue et al, 2008). Inducing rat-brain CYP2D with 7-day nicotine pretreatment (given 8 h ahead of codeine) increased brain morphine levels and analgesia, after an intraperitoneal codeine injection; this was reversed by pretreatment with i.c.v. propranolol (CYP2D inhibitor) (McMillan and Tyndale, 2015). Peak codeine analgesia correlated with brain, but not plasma, morphine levels and there was no effect of brain CYP2D inhibition or induction pretreatments on baseline nociception, peripheral codeine or morphine levels, or analgesia following a morphine injection (McMillan and Tyndale, 2015). These results together indicated that the early analgesic effects of peripherally administered codeine are dependent, in part, on brain CYP2D activation of codeine to morphine, prior to the entry into the brain of morphine produced by hepatic metabolism. While nicotine can elicit analgesia via nicotinic acetylcholine receptor (nAChR) activation, the timing of the pretreatment (ending 8 h before codeine), the lack of effect of this pretreatment paradigm on baseline nociception, or morphine-induced analgesia, and the reversibility of the nicotine pretreatment effect by i.c.v. propranolol argues against a direct effect of nicotine on analgesia in our antinociception paradigm (Tripathi et al, 1982). Whether the effect of brain CYP2D on codeine analgesia has implications beyond acute drug response, to the development of analgesic tolerance to codeine, and other opioid drugs metabolized by CYP2D, is unknown.

Brain CYPs have high sensitivity to environmental inducers compared to their hepatic forms (Ferguson and Tyndale, 2011; Miksys and Tyndale, 2013). Liver CYP2D is essentially un-inducible and variation is primarily due to genetics, whereas brain CYP2D levels can be altered by these same genetics, and also by environmental exposure to compounds such as nicotine (Miksys and Tyndale, 2004). Rat and monkey brain, but not liver, CYP2D is induced by
repeated exposure to nicotine, consistent with higher levels of human brain, but not liver, CYP2D in smokers (Mann et al., 2008; Mann et al., 2012; Miksys and Tyndale, 2004; Miller et al., 2014; Yue et al., 2008). Individuals with higher brain CYP2D may experience a faster onset of, and increased, analgesia from codeine, through earlier and greater brain morphine formation. This in turn could alter the rate of tolerance. Here, we sought to examine the effect of inducing brain CYP2D on repeated codeine exposure and the development of tolerance to codeine’s analgesic effects; we hypothesized that codeine tolerance would develop as a function of central morphine formation, in that inducing rat brain, but not hepatic, CYP2D in vivo would increase the rate of codeine tolerance during repeated daily oral codeine exposure. Rat CYP2D is a useful model of human CYP2D6-mediated codeine metabolism due to similarities in substrate specificity, and relative amount of morphine produced from codeine in vivo by rat CYP2D and human CYP2D6 (Hedenmalm et al., 1997; Hiroi et al., 2002; Oguri et al., 1990). This study aims to elucidate if variable rat brain CYP2D activity plays a significant part in chronically administered CNS-acting drug response, specifically codeine analgesic tolerance, thus extending our understanding of both tolerance, and the role of brain CYP enzymes in drug response.

3.3 Methods and Materials

**Animals**

Adult male Wistar rats (250–500 g; Charles River, St-Constant, Canada) were housed in triplets with ad libitum food and water. Food was restricted one hour prior to, and during, experimentation to decrease variability in oral drug absorption. Rats were maintained under a 12 h artificial light/dark cycle with experimentation occurring during the light cycle. All procedures were in accordance with the Canadian Council on Animal Care guidelines for the care and use of laboratory animals, and approved by the Animal Care Committee at the University of Toronto.

**Drug treatment**

Codeine phosphate and morphine sulfate (PCCA, London, Canada) were dissolved in saline and injected via oral-gavage (p.o.), a route of administration that incorporates first-pass metabolism and closely resembles the oral route of codeine dosing used by humans. The doses of codeine (40 mg/kg) and morphine (20 mg/kg) used in the brain CYP2D induction paradigm were chosen from dose–response curves, to obtain sub-maximal peak analgesia, capable of detecting
potential increases in analgesia, occurring due to brain CYP2D induction, or potential decreases due to tolerance. The subsequent higher doses of codeine (50 and 60 mg/kg) and morphine (25 and 30 mg/kg) were used as they resulted in incremental increases in peak analgesia relative to the initial doses of codeine and morphine used, extending the findings on initial analgesia and tolerance. Nicotine bitartrate (MP Biomedicals, Solon, OH) at 1 mg base/kg (in saline, pH 7.4) or saline vehicle at 1 ml/kg was given subcutaneously (s.c.) daily for seven days, a paradigm which results in induced rat brain, but not liver, CYP2D levels and activity (McMillan and Tyndale, 2015; Yue et al, 2008).

**Nociceptive testing of codeine and morphine analgesia**

Antinociception (denoted throughout as analgesia) was measured as the percentage of maximal possible effect (%MPE) by tail-flick latency using a tail-flick meter (Columbus Instruments, Columbus, OH), as previously described (D'Amour and Smith, 1941; McMillan and Tyndale, 2015). The thermal strength was adjusted to obtain baseline tail-flick latencies (TFL) between 3 and 4 s (lamp intensity 8.0, change in temperature (from room temperature) 〜45 °C); a cut-off of 10 s (〜3-times baseline) was used to avoid tissue damage. TFLs were measured twice in each animal 30 min prior to experimentation and the mean was used as the individual rat’s baseline TFL. [%MPE = (post-injection latency − baseline latency)/(maximum cut-off − baseline latency) × 100%].

**Tolerance experimental design**

The full experimental design is outlined in Figure 18. Nicotine or saline was administered for seven days prior to (‘Pretreatment’ period), and during (‘Treatment’ period) opioid administration. All opioid treatments were given at 8 h after the seventh, and every subsequent, nicotine or vehicle pretreatment. During the treatment period, test animals were given codeine (40 mg/kg; p.o.) or morphine (20 mg/kg; p.o.) daily; a full time-course of analgesia was assessed by TFL (every 5 min for 30 min and then every 10 min for the next 30 min) on treatment days 1 and 7, while analgesia was assessed at 30 min (peak analgesia on day 1) for the five treatment days in between (days 2–6). Full analgesia time-courses were not performed on days 2–6 to prevent behavioural adaptation as well as physical damage inflicted by repeated testing with the tail-flick apparatus. In control animals, codeine or morphine was given on treatment days 1 and 7 only, with vehicle injections
on the treatment days in between (days 2–6). Analgesia was assessed as performed in test animals. Animals were deemed tolerant when their post-injection 30 min TFL was reduced by 75% or more compared with the average 30 min TFL on treatment day 1, and was not statistically different from baseline TFL (Wang et al., 2005c). Codeine tolerance experiments were performed in a group of rats (n = 25), split by housing group among pretreatment (nicotine vs. vehicle) and treatment (test vs. control) group. This was reproduced in a subsequent separate group of rats (n = 25); data are displayed with both groups combined. Morphine tolerance, and additional codeine/morphine dose experiments, were run in two separate sets of rats (n = 24/experiment), again split by housing group for pretreatment (nicotine vs. vehicle), treatment (test vs. control), and/or dose.

**Figure 18: Experimental study design and timeline for pretreatment and treatment phases of tolerance experiment.** Rats in the codeine or morphine tolerance studies were randomized to receive either daily nicotine (brain CYP2D inducer) or vehicle pretreatment and to be run as a ‘Test’ or ‘Control’, as displayed.

**Plasma and brain sample preparation for drug level and enzyme activity analyses**

To further assess the impact of nicotine pretreatment on brain and liver CYP2D enzyme activity, as well as brain and peripheral codeine metabolism, a sub-set of rats in the test codeine paradigm (n = 12) were sacrificed by decapitation on treatment day 7 at 30 min (approximately peak analgesia). Trunk blood was centrifuged at 5000g for 10 min for plasma isolation. Whole brains (minus cerebellum) were homogenized in 0.01 N HCl (1:3 w/v) and centrifuged at 5000g for 10 min.

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Membrane preparation
Rat brain CYP2D is present and active in microsomal, plasma, mitochondrial, and nuclear membrane (Miksys et al., 2000b); total brain membrane fractions were prepared as outlined previously (Tyndale et al., 1999). In brief, fresh cerebellum (approximately 0.4 g) was homogenized in 6 ml artificial cerebrospinal fluid (ACSF, pH 7.4) and centrifuged at 3000g for 10 min. The supernatant was centrifuged at 110,000g for 60 min and the resulting pellet was re-suspended in 150 µl ACSF. Liver microsomes were prepared as previously described (Siu et al., 2006). Protein concentrations were measured using the Bradford assay with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Mississauga, Canada).

Ex-vivo dextromethorphan oxidation
Formation of dextrorphan from dextromethorphan was used to assess CYP2D enzyme activity using published methods with some modifications (Kerry et al., 1993; Tyndale et al., 1999). For brain dextromethorphan metabolism, freshly prepared membranes (1.5 mg protein/ml) were incubated with 50 µM dextromethorphan hydrobromide (Sigma-Aldrich, Oakville, Canada) and 1 mM NADPH in ACSF for 180 min at 37 °C under 95% O₂/5% CO₂ in a final volume of 1 ml. For hepatic dextromethorphan metabolism, microsomal membranes (0.5 mg protein/ml) were incubated with 2.5 µM dextromethorphan, 100 mM KH₂PO₄·H₂O buffer (pH 7.4) and 1 mM NADPH in dH₂O for 5 min at 37 °C in a final volume of 0.5 ml. The reactions were stopped with an equal volume of hexane-butanol (95:5 v/v). Controls included conditions without substrate and also without membrane protein.

Measurement of drugs
Dextromethorphan and dextrorphan were measured following brain membrane or liver microsome incubations through modified HPLC methods as described previously. Separate standard curves for dextromethorphan (50–5000 ng/ml) and dextrorphan tartrate (Sigma-Aldrich; 5–50 ng/ml) were used. In brief, samples were extracted into 5 ml hexane-butanol (Flores-Pérez et al., 2004) with 50 ng of 2-benzoxazolinone (Sigma-Aldrich; internal standard), the organic layer was collected and evaporated to dryness at 37 °C under nitrogen steam, and the residue was re-dissolved in mobile phase. Dextromethorphan (retention time 22.6 min, recovery rate 72.2%) and dextrorphan (10.5 min, 78.3%) standard curves, internal standard (13.3 min), and samples were
resolved at ambient temperature on an Agilent 1200 HPLC (Palo Alto, CA) equipped with a ZORBAX Bonus-RP column (250 mm × 4.6 mm I.D.; particle size, 5 μm; Agilent Technologies, Mississauga, Canada). Gradient elution conditions used solvent A (methanol and 0.05 M phosphate buffer, 45:55 v/v, pH = 5.8) and solvent B (HPLC grade water), 0–14 min 100–70% solvent A at 0.8 ml/min, 14–27 min 70–100% solvent A at 0.8–1.2 ml/min, 27–28 min at constant solvent A at 1.2–0.8 ml/min. Eluent fluorescence was monitored at an excitation wavelength of 230 nm and an emission wavelength of 330 nm (Hendrickson et al, 2003), with a limit of quantification of 0.5 ng for dextromethorphan and dextrophan. Codeine and morphine were quantified by HPLC as performed previously (McMillan and Tyndale, 2015; Zhou et al, 2013).

Statistical analyses
Between-animal study designs were used. Data were analyzed by repeated measures two-way ANOVAs followed by Bonferroni corrections for multiple comparisons, unpaired two-tailed t-tests, or Pearson correlations, as indicated in figure legends.

3.4 Results

Inducing brain CYP2D increased acute codeine analgesia and the rate of codeine tolerance; the rate of tolerance correlated with initial codeine analgesic response

On treatment day 1 after the first codeine administration, compared to vehicle, nicotine pretreatment resulted in significantly higher %MPE and overall area under the analgesia-time curve (AUC0–60 min; p < 0.05, n = 12–13/group; Figure 19A). Animals were given codeine daily for the following 6 days and analgesia was tested throughout; pretreatments were continued at 8 h prior to codeine administration. On treatment day 7 of codeine administration (day 14 of nicotine or vehicle pretreatment), animals showed low levels of analgesia (below 20%; Figure 19B). To assess the effect of brain CYP2D induction on the rate of codeine tolerance, analgesia was assessed at 30 min after daily codeine administration, the time at which analgesia peaked on treatment day 1. By treatment day 4 in both pretreatment groups, the majority of animals were tolerant, showing little change in analgesia from day 4 to day 7, therefore although both 1 to 7 and 1 to 4 were analyzed, the change in analgesia from treatment days 1 to 4 alone was deemed most relevant. Compared to vehicle pretreatment, nicotine pretreatment had a faster rate of codeine tolerance (p < 0.03; Figure 19C), presenting as a more rapid decrease (steeper slope, −10.0 vs. −3.8) in
codeine analgesia across treatment days 1–4. Individual animal analgesia on treatment day 1 (%MPE at 30 min) correlated with the rate of codeine tolerance (p < 0.001; Figure 19D), suggesting that initial codeine analgesic response predicted the rate of codeine tolerance (which was higher and faster in the brain CYP2D induced group). Animal baseline TFLs prior to codeine treatment did not change significantly across the treatment period (p > 0.7), suggesting no increased sensitization (hyperalgesia) across repeated codeine administration, or between pretreatment groups (p > 0.9), suggesting no effect of nicotine on baseline response.

Compared to vehicle pretreated rats (8.6 + 1.5 nmol/mg/h, mean + SEM), nicotine pretreated rats had significantly higher ex vivo brain CYP2D activity (13.5 + 1.3 nmol/mg/h; p < 0.05 sacrificed on treatment day 7 at 30 min after codeine administration) consistent with brain CYP2D induction by nicotine. There was no change in ex vivo hepatic CYP2D activity between vehicle (0.17 + 0.01 nmol/mg/min) and nicotine (0.19 + 0.03 nmol/mg/min; p > 0.6) pretreatment groups. Consistent with increased brain, but not liver, CYP2D enzyme activity, animals pretreated with nicotine trended towards a higher brain morphine/codeine ratio (0.18 + 0.05, mean + SEM, compared to 0.09 + 0.02; p < 0.2) with no impact on the plasma morphine/codeine ratio (0.49 + 0.09 compared to 0.41 + 0.18; p > 0.7), suggesting an effect of nicotine pretreatment on central, but not peripheral, metabolism of codeine to morphine.
Figure 19: Nicotine pretreatment increased acute codeine-induced analgesia; repeated codeine injections resulted in analgesic tolerance, the rate of which was increased by nicotine pretreatment. Compared with vehicle, nicotine pretreatment increased %MPE and analgesic AUC 0-60 min (a) after acute codeine administration (40 mg/kg, p.o.; n = 12–13/group); repeated daily codeine administration resulted in analgesic tolerance (b). Compared to vehicle, nicotine pretreatment increased the rate of onset of codeine tolerance (c); individual animal peak analgesia on day 1 (at 30 min) correlated to the decrease in analgesia between days 1–4 (d). *p < 0.05 and **p < 0.01 using repeated measures two-way ANOVA with Bonferroni post-hoc test and t-tests. For correlations, Pearson coefficients were used.

Effect of brain CYP2D induction was maintained and there was no significant change in analgesia after a one-week washout between codeine administrations

To determine whether the changes in analgesia across repeated codeine injections were due to tolerance to codeine’s analgesic effect, control groups receiving all pretreatments, but daily vehicle instead of codeine injections on treatment days 2–6, were assessed. This also tested the extent to which vehicle injections on treatment days 2–6 and daily analgesia testing sensitized animals to
the behavioural assay. As a replication of the findings in Figure 19A, after acute codeine administration compared to vehicle pretreatment, nicotine pretreatment resulted in significantly higher %MPE and AUC$_{0-60}$ min ($n = 12–13$/group; Figure 20A) as seen in test codeine animals (Figure 19A). On treatment day 7, after one-week without codeine, the effect of nicotine-mediated induction of brain CYP2D on codeine analgesia remained; animals pretreated with nicotine had higher %MPE and AUC$_{0-60}$ min (Figure 20B) after codeine than vehicle pretreated animals. After each vehicle administration on treatment days 2–6 (Figure 20C) there was no significant difference in %MPE between pretreatment groups ($p > 0.4$ across all days) or TFLs compared to baseline ($p > 0.2$). Comparing codeine response on treatment days 1 and 7, there was no correlation between individual animal peak analgesia on day 1 (%MPE at 30 min) and change in response from day 1 to day 7 (flat slope; Figure 20D), suggesting no effect of repeated vehicle injections and testing on codeine induced analgesia.

**Inducing brain CYP2D did not affect acute morphine analgesia or the rate of morphine analgesic tolerance; there was no change in morphine analgesia after a one-week washout**

Morphine is the active analgesic metabolite of codeine and is not a substrate of CYP2D; thus, inducing brain CYP2D had no effect on acute morphine analgesia (McMillan and Tyndale, 2015) and should not alter the rate of morphine tolerance. On treatment day 1 after acute morphine administration, compared to vehicle pretreatment, nicotine pretreatment did not alter %MPE or AUC$_{0-60}$ min ($n = 6–7$/group; Figure 21A). On treatment day 7, animals showed negligible analgesia and there remained no effect of nicotine pretreatment on %MPE and AUC$_{0-60}$ min (Figure 21B). Similar to the codeine experiment (Figure 19C), the majority of test animals in both pretreatment groups experienced tolerance by treatment day 4 with negligible remaining analgesia and little change through day 7. Analgesia decreased at the same rate in both pretreatment groups (similar slopes, $p > 0.9$; Figure 21C) from treatment days 1–4 of morphine administration. Similar to codeine, individual animal analgesia on day 1 (%MPE at 30 min) correlated with the rate of morphine tolerance ($p < 0.001$; Figure 21D), suggesting that initial response to codeine or morphine predicted the rate of tolerance; the higher initial analgesia, the faster the decline in analgesia.
Figure 20: Control animals showed no change in analgesia after a one-week washout between codeine injections; analgesia was not altered by daily p.o. (i.e. codeine vehicle) injections or behavioural testing. Compared with vehicle, nicotine pretreatment increased %MPE and analgesic AUC_{0-60 min} (a) after acute codeine administration (40 mg/kg, p.o.; n = 12–13/group) as expected and observed previously (Fig. 19a); the effect of nicotine pretreatment on codeine analgesia was sustained after a one-week washout (b). There was no effect of p.o. vehicle injections on %MPE across days 2–6 (c); negative slope shows slight decrease in analgesia between codeine injections one-week apart (d). *p < 0.05, **p < 0.01 and ***p < 0.001 using repeated measures two-way ANOVA with Bonferroni post-hoc test and t-tests. For correlations, Pearson coefficients were used.
Figure 21: Nicotine pretreatment did not alter morphine-induced analgesia; repeated morphine injections resulted in analgesic tolerance, the rate of which was not altered by nicotine pretreatment. Compared with vehicle, nicotine pretreatment did not alter %MPE and analgesic AUC$_{0-60}$ min (a) after acute morphine administration (20 mg/kg, p.o.; n = 6/group); repeated daily morphine administration resulted in analgesic tolerance (b). Compared to vehicle, nicotine pretreatment did not alter the rate of onset of morphine tolerance (c); individual animal peak analgesia on day 1 (at 30 min) correlated to the decrease in analgesia between days 1–4 (d). Repeated measures two-way ANOVA with Bonferroni post-hoc test, t-tests and Pearson coefficients were used.

In control animals, compared to vehicle pretreatment, nicotine pretreatment did not alter %MPE or AUC$_{0-60}$ min (n = 6–7/group; Figure 22A) after acute morphine administration on day 1, or morphine on day 7 after a one-week washout (Figure 22B). Similar to codeine control animals (Figure 20), there was no significant difference in %MPE between pretreatment groups (p > 0.3 across all days) or TFLs compared to baseline (p > 0.7) after vehicle administration on treatment days 2–6 (Figure 22C), as well as no correlation between individual animal peak analgesia on day
1 and change in response after washout (flat slope; Figure 22D), overall suggesting no effect of repeated vehicle injections and testing on morphine induced analgesia.

**Figure 22:** Control animals showed no change in analgesia after a one-week washout between morphine injections; analgesia was not altered by daily p.o. (i.e. morphine vehicle) injections and behavioural testing. Compared with vehicle, nicotine pretreatment did not alter %MPE and analgesia AUC$_{0-60}$ min(a) after acute morphine administration (20 mg/kg, p.o.; n = 6/group); analgesia was sustained after a one-week washout (b). There was no effect of p.o. vehicle injections on %MPE across days 2–6 (c); negative slope shows slight decrease in analgesia between morphine injections one-week apart (d). Repeated measures two-way ANOVA with Bonferroni post-hoc test, t-tests and Pearson coefficients were used.

**Rate of tolerance correlated directly to initial analgesic response, independent of pretreatment and codeine or morphine dose**

To further assess the relationship between increasing initial analgesia and development of tolerance to codeine and morphine, a group of animals without pretreatments (i.e. unaltered brain
CYP2D activity) were given higher doses of daily codeine (50 or 60 mg/kg; p.o.) and morphine (25 or 30 mg/kg, p.o.). Increased doses of codeine and morphine resulted in significantly higher day 1%MPE and analgesic AUC (p < 0.001 and p = 0.05, respectively) using two-way ANOVA. As seen previously (Figures 19 and 21), with daily dosing, these animals became tolerant by treatment day 4 (Fig. 23C); individual animal peak-analgesia on day 1 (%MPE at 30 min) correlated with the rate of tolerance (p < 0.001; Figure 23D). Combining the data from codeine and morphine alone (Fig. 23), with data following brain CYP2D induction (Figures 19 and 21), confirmed that the initial peak analgesia from acute exposure (%MPE at 30 min on treatment day 1) correlated with the mean rate of tolerance from treatment days 1–4 in all test groups (p < 0.001; Figures 24A and B). This correlation remained significant when comparing initial peak analgesia to slope from day 1 to day 7 (R = 0.95, p < 0.001). This again confirmed that acute day 1 initial analgesic response predicted the rate of tolerance and that the pretreatments simply altered the magnitude of initial analgesic response. Codeine, via CYP2D metabolism to morphine, and morphine, via direct action, elicit analgesia through morphine, suggesting a relationship between morphine action at the opioid receptor and the rate of tolerance.
Figure 23: Initial analgesia correlated to rate of tolerance in animals given higher doses of codeine and morphine. Acute codeine (50 or 60 mg/kg, p.o.) and morphine (25 or 30 mg/kg; p.o.) administration produced peak analgesia in a dose dependent manner (n = 5–6/group, p < 0.001 and p = 0.05 compared to earlier doses of codeine and morphine, respectively; a); repeated daily administration resulted in analgesic tolerance (b). Rate of tolerance (slope from day 1 to 4) was dose dependent (c) and correlated to peak analgesia after acute administration on day 1 (d). *p < 0.05, **p < 0.01 between codeine doses and #p < 0.01 between morphine doses using two-way ANOVA with Bonferroni post-hoc. Pearson coefficients were used.
Figure 24: Initial analgesia predicts the rate of onset of tolerance across multiple codeine and morphine doses. Peak analgesia after the first codeine or morphine injection correlated to the change in peak analgesia across the first four days of codeine (n = 12–13/group; n = 5–6/group for higher doses) or morphine (n = 6/group; n = 5–6/group for higher doses) administration, regardless of dose (Codeine: 40, 50, and 60 mg/kg p.o.; Morphine: 20, 25, and 30 mg/kg p.o.) or pre-treatment (seven-day nicotine, seven-day vehicle, or no pretreatment). Data are displayed as group means (a) and individual animals (b). Pearson correlations were used.

3.5 Discussion
This is the first study to show that nicotine-induced brain CYP2D metabolism of codeine to morphine can alter the response to repeated codeine (i.e. tolerance), providing evidence that brain CYP-mediated metabolism can impact chronically administered CNS-acting drug effects. During chronic daily opioid induced analgesia, induced brain CYP2D activity increases initial codeine analgesia, resulting in a faster rate of codeine induced analgesic tolerance. Further, it demonstrated that increasing initial analgesia, from either codeine or morphine, increased the rate of tolerance (decrease in peak analgesia across days) with no effect on time to full tolerance. Inducing brain CYP2D with nicotine increased initial codeine analgesia, and resulted in a significantly greater rate of decrease in analgesia across the first four days of treatment (steeper slope), compared to vehicle pretreated animals that had less initial codeine analgesia, and a comparatively decreased rate of tolerance (shallower slope). The rate was increased, rather than
resulting in an equal rate of tolerance but longer sustained analgesia in animals with a higher initial analgesic response. In animals given daily morphine (not a substrate of CYP2D), there was no effect of nicotine induction of brain CYP2D on initial morphine analgesic response or on the rate of tolerance. The relationship between initial analgesia and tolerance was further supported in animals without brain CYP2D pretreatments, where increased codeine and morphine doses administered increased initial analgesia, and thereby resulted in an increase in the absolute drop in analgesia, or rate of tolerance, but not in the time to reach tolerance (i.e. days to negligible analgesia). These findings are related to a 1969 paper which indicated that degree of tolerance to levorphanol-induced running-fit in mice was related to its original (first injection) intensity (Goldstein and Sheehan, 1969). This finding suggested a link between acute and chronic opioid response, using a distinct animal model of excitatory opioid action; we are unaware of any publications illustrating the relationship between initial analgesia and rate of analgesic tolerance to opioids.

The initial analgesic response and rate of tolerance correlated in all animals assessed, independent of brain CYP2D activity (vehicle or nicotine pretreatment) or dose of compound (codeine or morphine), confirming that the rate of tolerance was related to initial analgesic response. In all experimental conditions, the majority of test animals experienced analgesic tolerance by the fourth opioid administration, which is interesting to consider as it relates to the rate of codeine tolerance with and without nicotine-induced brain CYP2D. Nicotine pretreated animals experienced a similar proportional daily decrease (28% per day) in codeine analgesia to vehicle pretreated animals (33%), however the absolute drop was greater (11.2%MPE per day vs. 4.7%MPE). In individuals who experience greater initial pain relief, a 50% decrease in efficacy of an opioid may present as a greater, or more noticeable, drop in drug efficacy than those who experience less initial analgesia. Thus, while the drop is proportional, for those with higher initial analgesia, the absolute drop is greater. A greater drop in response could, in turn, lead to a greater motivation to seek better pain control resulting in quicker or more pronounced dose-escalation. In individuals with cancer pain challenged with a morphine dose before and after one week of regular morphine treatment, a ∼1.5-fold greater morphine dose was required to achieve the same degree of pain relief (Houde and Beaver, 1966). Our findings suggest that a greater magnitude of response from the first morphine challenge, potentially resulting in more rapid tolerance, can result in dose escalation in
a shorter time frame than one week. In support of this, individuals treated by “high-dose” emergency physicians showed significantly increased odds of long-term opioid use compared to those treated by “low-dose” emergency physicians (and “high-intensity” compared to “low-intensity” physicians) (Barnett et al, 2017). Chronic opioid dose escalation in pain patients and illicit opioid users is well documented; our findings on the impact of altered brain CYP2D metabolic activation of codeine shed some light on one potential mechanism which might motivate differential dose escalation (Collett, 1998; Cowan et al, 2001).

These findings provide one possible mechanistic link for an impact of smoking on increasing codeine dependency. The nicotine pretreatment paradigm used in this study resulted in average daily nicotine plasma levels similar to those seen in human smokers and experienced electronic cigarette (e-cigarette) users (20–50 ng/ml) (Farsalinos et al, 2015; McMillan and Tyndale, 2015; Velez de Mendizabal et al, 2015); similar to nicotine-treated animals, smoking is associated with higher human brain, but not hepatic, CYP2D (Miksys and Tyndale, 2004). Studies assessing the impact of smoking on opioid tolerance are often conducted in chronic, and post-operative, pain situations where findings are inconsistent; smoking has been found to increase, decrease and have no effect on opioid escalation across time (Chiang et al, 2016; Skurtveit et al, 2010; Weingarten et al, 2011). However, the acute and chronic effects of nicotine on nociception, pain sensitivity, and interaction with the wide-range of opioids with analgesia affected (e.g. codeine) or unaffected (e.g. morphine) by potential changes in CYP2D activity, is complex and multifactorial (Girdler et al, 2005). The effect of nicotine on increasing brain CYP2D-mediated codeine metabolic activation and analgesia, and the resulting acceleration in the rate of analgesic tolerance could contribute to potential dose-escalation, which may increase the prevalence of codeine-dependency among smokers.

One concern with continued nicotine pretreatment throughout the treatment phase, was the potential interaction of nicotine with codeine and morphine-induced analgesia. Nicotine can play a role in inhibitory-pain control, and can elicit analgesia in animal models through the involvement of endogenous opioid transmission (Berrendero et al, 2002). Nicotine and opioids also exhibit cross-tolerance or cross-sensitization (Vihavainen et al, 2008; Zarrindast et al, 2003). We have previously shown, as well as confirmed here, that under the current nicotine dosing regime,
just prior to codeine or morphine administration, nicotine levels are undetectable (McMillan and Tyndale, 2015). We also showed that nicotine pretreatment did not alter pre- or post-injection baseline latencies (suggesting no effect on locomotion), ex vivo hepatic CYP2D activity and hepatic morphine/codeine ratios in codeine test animals, nor did it have an effect on acute morphine analgesia, or the rate of morphine tolerance. This is consistent with a lack of direct nicotine effect itself, on opioid-induced analgesia, via nAChR activation or otherwise. We saw an effect of nicotine pretreatment on ex vivo brain CYP2D activity but failed to see a significant change on brain morphine/codeine ratio; ratios were examined in whole brain homogenates (minus cerebellum), which may have blunted the ability to see regional impacts expected due to the heterogeneity in CYP2D expression and induction (Miksys and Tyndale, 2004; Yue et al, 2008). An additional limitation of this study is in modelling human brain CYP2D6 with rat brain CYP2D; while CYP2D6 is the only functional CYP2D isoform in humans, six homologous isoforms have been identified in rats which show some variation in substrate specificity and tissue expression (Haduch et al, 2011; Hiroi et al, 2002; Hiroi et al, 1998a). Rat CYP2D isoforms and human CYP2D6 are expressed in the brain, catalyze the O-demethylation of codeine to morphine and dextromethorphan to dextrorphan, and mediate the mechanism-based (suicide) inhibition by propranolol (Grobe et al, 2012; Hiroi et al, 2002; Hiroi et al, 1998a; Rowland et al, 1994; Zanger et al, 2004). In addition, both rat and non-human primate (i.e. African Green Monkey) brain, but not liver, CYP2D are induced by nicotine in vivo consistent with the higher brain, but not liver, CYP2D in human smokers (Mann et al, 2008; Miksys et al, 2002; Miller et al, 2014; Yue et al, 2008; Zanger and Schwab, 2013). Together this suggests that while the precise isoform involved is unknown, the rat is useful for modelling these CNS aspects of human brain CYP2D6. The tail-flick reflex assay is a reliable and reproducible assay indicating spinal and supra-spinally mediated analgesia; the tail-flick reflex also shows little inter-animal variability in baselines or with repeated testing, rarely occurs spontaneously, and can predict the analgesic effects of opioids in humans (Grossman et al, 1982; Le Bars et al, 2001). We have previously shown that nicotine induction of brain CYP2D resulted in an increase in codeine, but not morphine, analgesia measured by the hot-plate assay (McMillan and Tyndale, 2015). Lastly, the mechanism of induction of brain, but not hepatic, CYP2D expression with chronic nicotine pretreatment is currently unknown, but occurs in the absence of changes in mRNA suggesting it may be mediated by increased enzyme stability or decreased degradation (Yue et al, 2008).
The implications of our findings are that individuals with higher brain CYP2D activity (e.g. genetically ultra-rapid CYP2D metabolizers or smokers), those taking larger initial doses, or those more sensitive to opioids via an alternative mechanism (e.g. greater mu-opioid receptor availability) may experience a greater initial response to, and therefore more rapid rate of tolerance to, codeine. The influence of brain CYP2D metabolism on acute codeine response has been observed with tramadol, another opioid analgesic metabolized by CYP2D. Selective rat brain CYP2D inhibition and induction decreased and increased, respectively, central tramadol metabolism and analgesia (Wang et al, 2015). This is further evidence that altered brain CYP2D metabolism alters acute opioid metabolic activation and analgesia, suggesting the potential for altering tolerance to tramadol response and potentially to other CYP2D6 substrate opioids including oxycodone and hydrocodone (Hutchinson et al, 2004; Lalovic et al, 2006). Beyond genetics and smoking, there are numerous environmental and endogenous factors that can further contribute to variable brain CYP2D, including hormonal, sex and developmental factors (Wang et al, 2014). Monkeys self-administering ethanol had a dose-dependent increase in brain, but not liver, CYP2D, consistent with increased brain CYP2D levels in human alcoholics (Miksys et al, 2002; Miller et al, 2014). The neuroleptic clozapine and neurotoxin toluene have also been shown to induce rat brain CYP2D (Hedlund et al, 1996). While sex-based differences in brain CYP2D activity and expression remain unclear, sex hormones play a role in regulating expression of brain CYP2D; testosterone and progesterone produced a marked increase and decrease, respectively, of brain CYP2D mRNA levels female rats (Aichhorn et al, 2005; Baum and Strobel, 1997; Bergh and Strobel, 1996; Hildebrandt et al, 2003; Li et al, 2015a; Waxman and Holloway, 2009). Furthermore, the expression of human brain CYP2D6 increases with age (Mann et al, 2012). Thus, being a smoker, having rapid CYP2D genetics, taking higher initial doses, or any prior-mentioned cause for induced brain CYP2D metabolism may increase the rate of tolerance and risk of dose escalation and/or dependence, providing an argument for lower initial doses in pain treatment. Likewise, even with repeated morphine administration, the higher initial response predicted a faster rate of tolerance suggesting that even among non-enzymatically activated opioids initiating pain relief with the lowest possible doses may be prudent.
This study provides a novel mechanistic approach to understanding the potential role of central metabolism on individual differences in pain relief and tolerance to codeine and potentially other CYP2D substrate opioids (e.g. hydrocodone and tramadol). To our knowledge there has been little investigation of whether initial analgesia predicts tolerance, and if it does, whether initial analgesia is related to a proportional drop in analgesia (as seen by a change in slope) or an absolute drop in analgesia (similar slopes, and thus a longer time to tolerance in those with higher initial analgesia). Codeine remains a commonly abused opioid analgesic, largely due to its use in prescription and over-the-counter analgesic and cough syrup combinations (Cooper, 2013; Roussin et al, 2013). Codeine use also extends beyond prescription; in the US in 2014, 64.3 million people reported some lifetime non-medicinal opioid use (19.4 million attributed to codeine products), with 12.5 million people reporting current opioid misuse in 2015 alone (NSDUH, 2016, 2017). However, most opioid drugs are metabolized by CYP2D to compounds that are more potent µ-opioid receptor agonists than the parent opioid, suggesting that the findings from this study provide a proof-of-concept that could extend beyond codeine to other commonly abused opioid analgesics (e.g. hydrocodone and tramadol). With the ongoing rise in risk of opioid use, abuse, dependency and overdose, the role of opioid metabolism within the brain, and variability in this metabolism, may be a novel source of inter-individual differences in opioid response and tolerance.

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3.7 Significance to Thesis
This original article expands on our findings of an impact of brain CYP2D metabolism on acute codeine administration, by showing a novel role for brain CYP2D in the development of codeine analgesic tolerance after repeated codeine administration. This study used oral gavage, a more clinically relevant route of codeine administration, and replicated findings from our first study that inducing rat brain CYP2D increased initial codeine analgesia. This increase in initial codeine analgesia resulted in an increased the rate of codeine analgesic tolerance following repeated codeine administration. This increased rate of tolerance presented as a greater absolute drop in codeine analgesia (similar proportional drop) per oral injection. Furthermore, this study replicated findings that inducing brain CYP2D did not alter morphine-induced initial analgesia (not a CYP2D substrate), again using oral gavage administration, and as expected found no impact of inducing brain CYP2D on the rate of morphine tolerance across repeated morphine administration. This study showed that the rate of codeine or morphine tolerance correlated to initial peak analgesia after the first codeine or morphine administration. This relationship was observed when initial codeine, but not morphine, analgesia was increased by inducing brain CYP2D and also when initial codeine and morphine analgesia was increased by giving greater doses.

We demonstrated here for the first time that initial analgesia 1) is related to the rate of tolerance, and 2) does not change the time to reach tolerance. Increasing initial analgesia produced a greater absolute decrease in analgesia or rate of tolerance (slope) but similar time to tolerance (4 days in all groups) rather than a similar absolute decrease in analgesia and therefore longer time to tolerance. The clinical implications of these findings are that individuals who experience greater initial opioid effect would experience a more dramatic decrease in drug effect with repeated opioid taking. The consequence of a greater perceived decrease in opioid-induced analgesia over time may be that these individuals are more likely to increase opioid dose to achieve the initial level of analgesia, and therefore they may be more vulnerable to the associated risks of higher opioid doses. Consequently, this study provides a further rationale for initiating opioid therapy at the lowest analgesic doses possible.
4. Chapter 3: Rat brain CYP2D activity alters in vivo central oxycodone metabolism, levels, and resulting analgesia

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Dr. Rachel F. Tyndale, Dr. Sharon Miksys and Douglas M. McMillan designed the overall study. DMM 1) conducted all analgesia, microdialysis, and ex vivo metabolism experiments, 2) performed the data analysis, and 3) wrote the manuscript. SM performed some pilot studies. Fariba Baghai Wadji assisted in animal cannulation surgeries and optimizing the microdialysis assay for oxycodone and metabolite detection. Dr. Bin Zhao developed and performed the LCMS assay for quantification of oxycodone and metabolites. RFT guided the manuscript writing and RFT and SM edited the manuscript.
4.1 Abstract

Oxycodone is metabolized by CYP2D to oxymorphone. Despite oxymorphone being a more potent opioid-receptor agonist, its contribution to oxycodone analgesia may be minor because of low peripheral production, low blood-brain barrier permeability and central nervous system efflux. CYP2D metabolism within the brain may contribute to variation in central oxycodone and oxymorphone levels, thereby affecting analgesia. Brain CYP2D expression and activity are subject to exogenous regulation; nicotine induces rat brain, but not liver, CYP2D consistent with higher brain CYP2D in smokers. We assessed the role of rat brain CYP2D in orally administered oxycodone metabolism (in vivo brain microdialysis) and analgesia (tail-flick test) by inhibiting brain CYP2D selectively with intracerebroventricular propranolol (mechanism-based inhibitor) and inducing brain CYP2D with nicotine. Inhibiting brain CYP2D increased brain oxycodone levels (1.8-fold; \( P < 0.03 \)) and analgesia (1.5-fold AUC\(_{0-60}\); \( P < 0.001 \)) after oxycodone, while inducing brain CYP2D increased brain oxymorphone levels (4.6-fold; \( P < 0.001 \)) and decreased analgesia (0.8-fold; \( P < 0.02 \)). Inhibiting the induced brain CYP2D reversed the change in oxycodone levels (1.2-fold; \( P > 0.1 \)) and analgesia (1.1-fold; \( P > 0.3 \)). Brain, but not plasma, metabolic ratios were affected by pre-treatments. Peak analgesia was inversely correlated with ex vivo brain (\( P < 0.003 \)), but not hepatic (\( P > 0.9 \)), CYP2D activity. Altering brain CYP2D did not affect analgesia from oral oxymorphine (\( P > 0.9 \) for AUC\(_{0-60}\) across all groups), which is not a CYP2D substrate. Thus, brain CYP2D metabolism alters local oxycodone levels and response, suggesting that people with increased brain CYP2D activity may have reduced oxycodone response. Factors that alter individual oxycodone response may be useful for optimizing treatment and minimizing abuse liability.
4.2 Introduction

Oxycodone is a semisynthetic opioid analgesic widely used to treat moderate to severe pain (Riley et al, 2008), with analgesic efficacy for neuropathic (e.g. diabetic neuropathy), somatic (e.g. osteoarthritis), visceral (e.g. myocardial ischemia) and cancer pain (Riley et al, 2008). Despite recent efforts to reduce both oxycodone prescribing and misuse with the development of abuse-deterrent delivery formulations, oxycodone abuse remains a major public health concern (Webster, 2009). In 2015, in all Americans over the age of 12 years, approximately 28 million reported oxycodone use, and 4.3 million (15.3 percent of users) reported oxycodone misuse (NSDUH, 2016). In these individuals, oxycodone-containing products accounted for 29 percent of total opioid use and 34 percent of opioid misuse (NSDUH, 2016). Elucidating potential sources of variation in opioid response remains important for understanding individual differences in opioid analgesic response and risk of abuse.

Oxycodone is metabolized by cytochrome P450 (CYP) enzymes to three primary metabolites: oxymorphone (by CYP2D6 in humans; 11 percent of urinary recovery), noroxycodone (by CYP3A4 in humans; 45–47 percent of urinary recovery) and noroxymorphone (by CYP3A4 and CYP2D6 in humans; 14 percent of urinary recovery) (Klimas et al, 2013; Lalovic et al, 2006; Lalovic et al, 2004). Parent opioid and/or metabolite contribution to analgesia depends on numerous factors including target receptor affinity (primarily the μ-opioid receptor), proportional peripheral metabolism, influx across the blood–brain barrier (BBB) relative to efflux from the central nervous system (CNS) and concentrations in the brain (Bostrom et al, 2005; Mercer and Coop, 2011); the relative contribution of oxycodone and its metabolites to oxycodone analgesia is unclear (Lalovic et al, 2006).

Cytochrome P450 enzymes are present and active in the brain and can be induced independently from hepatic CYPs (Miksys and Tyndale, 2013). While hepatic CYP2D levels are regulated primarily by genetics, brain CYP2D expression may be altered by genetics, age and environmental exposures such as to smoking and nicotine (Miksys and Tyndale, 2004). Modifying rat brain-specific CYP2D enzyme activity in vivo can alter the central metabolism and effect of CNS-acting CYP2D substrates, including opioids (e.g. codeine and tramadol) (McMillan and Tyndale, 2015; Wang et al, 2015). Codeine requires activation by CYP2D to morphine to produce analgesia;
individuals who are genetically CYP2D poor metabolizers (lacking CYP2D6 activity), or who are given CYP2D inhibitors, produce little-to-no morphine and exhibit little-to-no codeine analgesia (Chen et al, 1991b; Sindrup et al, 1992; Sindrup et al, 1990). Selectively inhibiting and inducing rat brain CYP2D decreased and increased, respectively, brain, but not plasma, morphine levels and analgesia after codeine administration (McMillan and Tyndale, 2015). Unlike codeine, which is essentially inactive, oxycodone is a potent μ-opioid receptor agonist, and compared with its CYP2D metabolite oxymorphone, oxycodone is present at higher concentrations and has a higher partition across the BBB (rat brain/plasma ratio of 2.07 versus 0.23 at 60 minutes after intragastric oxycodone) (Bostrom et al, 2008; Lalovic et al, 2006). Therefore, while the influence of brain CYP2D metabolism on codeine analgesia is via metabolic activation, the influence of brain CYP2D metabolism on oxycodone analgesia is unclear and likely multifactorial. Brain CYP2D metabolism may contribute to oxycodone analgesia through formation of oxymorphone, which has a greater μ-opioid receptor affinity than oxycodone, may reduce oxycodone analgesia through local oxycodone metabolism or may have no impact on oxycodone analgesia (Klimas et al, 2013).

Our objective was to investigate the central CYP2D-mediated metabolism of oxycodone to oxymorphone and resulting analgesia, by selectively inhibiting and/or inducing rat brain CYP2D activity. The pre-treatments used to manipulate brain CYP2D metabolism have been shown to modify brain-specific CYP2D activity without altering (1) hepatic CYP2D, (2) baseline nociception or (3) antinociception from non-CYP2D substrates (e.g. morphine-induced analgesia) (McMillan and Tyndale, 2015; Miksys et al, 2017). Because of the possible opposing impacts of brain CYP2D on oxycodone analgesia as described previously, we used in vivo brain microdialysis to assess brain oxycodone and major metabolite levels. This enabled assessment of the relationship over time between brain CYP2D-mediated oxycodone metabolism, resulting oxycodone and oxymorphone levels, and oxycodone analgesia.

4.3 Materials and Methods

Animals

Male Wistar rats (Charles River Laboratories, Saint-Constant, Canada) were house in triplets with ad libitum food and water, under a 12-hour artificial light/dark cycle with experimentation occurring during the light cycle. Adult animals were used; over the duration of the experiments,
ages ranged from 8 to 14 weeks old and weights from 250 to 500 g. All procedures were conducted in accordance with the Canadian Council on Animal Care guidelines for the care and use of laboratory animals and approved by the Animal Care Committee at the University of Toronto.

**Drug treatment**

Oxycodone hydrochloride (Professional Compounding Centers of America, London, Canada) and oxymorphone hydrochloride (Toronto Research Chemicals Inc., Toronto, Canada) were dissolved in dH₂O and administered by oral gavage (p.o.). Selection of doses is outlined in Figure 25. Propranolol hydrochloride (Sigma-Aldrich, Oakville, Canada) was dissolved in 20 percent (w/v) 2-hydroxypropyl-β-cyclodextrin (cyclodextrin, Sigma-Aldrich) in dH₂O to deliver 20 µg base in 4 µl intracerebroventricularly (i.c.v.) 24 hours prior to oxycodone (or oxymorphone) administration (McMillan and Tyndale, 2015; Zhou et al, 2013). Propranolol is a CYP2D mechanism-based inhibitor (Narimatsu et al, 2001); in rats, i.c.v. propranolol pre-treatment inhibits brain, but not liver, *ex vivo* dextromethorphan and codeine metabolism, indicative of *in vivo* brain, but not liver, irreversible CYP2D inhibition (Miksys et al, 2017; Zhou et al, 2013). To deliver propranolol or vehicle i.c.v., guide cannulae were surgically implanted into the right lateral cerebral ventricle, and fixed with adhesive for repeated use, as previously described (McMillan and Tyndale, 2015; Miksys et al, 2017; Zhou et al, 2013). Compounds (e.g. mechanism-based inhibitors and dyes) injected i.c.v. into one lateral ventricle in rats have been shown to spread bilaterally throughout all regions of the brain (DeVos and Miller, 2013; Miksys and Tyndale, 2009). Propafenone hydrochloride (Sigma-Aldrich), a CYP2D competitive inhibitor (Xu et al, 1995), was dissolved in 20 percent cyclodextrin (Sigma-Aldrich) in dH₂O to deliver 40 µg base in 4 µl i.c.v. 30 minutes prior to oxycodone administration. Seven days of nicotine pre-treatment induces rat brain, but not liver CYP2D, consistent with higher brain CYP2D levels found in human smokers (Mann et al, 2008; Miksys et al, 2002; Yue et al, 2008); nicotine pre-treatment increases *in vivo* activation of codeine to morphine and *ex vivo* dextromethorphan metabolism in brain, but not liver, indicative of increased CYP2D activity (McMillan and Tyndale, 2015; Miksys et al, 2017). Nicotine bitartrate (MP Biomedicals, Solon, OH, USA) was given subcutaneously (s.c.) at 1 mg base/kg (in saline, 0.9 percent NaOH; pH 7) daily for 7 days, and oxycodone (or oxymorphone) was given at 8 hours after the last nicotine injection, when induction of brain
CYP2D is maximal and nicotine has been cleared from both the central and peripheral systems (Yue et al., 2008).

Figure 25: Oxycodone and oxymorphone doses were chosen from dose–response curves. Oxycodone (OC) at 12.5 mg/kg p.o. \((n = 13)\) and oxymorphone (OM) at 10 mg/kg p.o. \((n = 10)\) produced analgesic peaks of 62 and 70 percent, respectively, similar to the doses of codeine (COD, 20 mg/kg i.p.; 64 percent) and morphine (MOR, 3.5 mg/kg i.p.; 69 percent) used in a previous study assessing the impact of brain CYP2D metabolism on COD metabolism and analgesia. The COD and MOR i.p. dose–response data were previously published by (McMillan and Tyndale, 2015). %MPE, percentage of maximal possible effect; SEM, standard error of the mean.

Nociceptive testing of analgesia

Antinociception (denoted throughout as analgesia) was measured as the percentage of maximal possible effect \((%\text{MPE} = \frac{\text{postinjection latency} - \text{baseline latency}}{\text{maximum cut-off} - \text{baseline latency}} \times 100\%\) of tail-flick latency (TFL) using a tail-flick meter (Columbus Instruments, Columbus, OH, USA), as previously described (McMillan and Tyndale, 2015). The thermal strength was adjusted to obtain baseline TFLs between 3 and 4 seconds; a cut-off of 10 seconds was used to avoid tissue damage. Individual baseline TFLs were the average of two measurements prior to experimentation.
Analgesia experimental design
Full analgesia paradigms, including pre-treatment timing, are outlined in Supplementary (Figure 30). Food was restricted 1 hour prior to, and during, experimentation to decrease variability in oral drug absorption. After oxycodone or oxymorphone administration, analgesia was assessed over time, focussing on 0–60 minutes where oxycodone and oxymorphone analgesia peak (TFL timepoints: 5, 10, 15, 20, 25, 30, 40, 50, 60, 75 and 90 minutes).

Microdialysis cannulation and procedure
Rats were surgically implanted with guide cannulae (MD-2250, Bioanalytical Systems, Inc., West Lafayette, IN, USA) into the right nucleus accumbens shell (anteroposterior: +2.2 mm; mediolateral: −1.0 mm; and dorsoventral −5.8 mm from Bregma (Nelson et al, 2011)), as previously described (Garcia et al, 2016). Animals recovered for 7 days before beginning pre-treatment, and were housed singly with access to food, restricted to 20–25 g/day to minimize weight gain. A within-animal study design was used, where rats received each of four pre-treatments (vehicle, propranolol, nicotine, or nicotine and propranolol, as previously described) in randomized order (n = 9). Animals were connected to the microdialysis probes (concentric silica-coated, 2-mm membrane; MD-2200, Bioanalytical Systems, Inc.) and allowed to acclimate for at least 15 minutes while perfusion medium (Ringer's solution; 147 mM Na$^+$, 2 mM Ca$^{2+}$, 4 mM K$^+$, 155 mM Cl$^-$, pH 6.0) was pumped at a flow rate of 2 μl/min. Baseline dialysate was collected on ice in three intervals of 15 minutes (45 minutes total) prior to oxycodone administration (12.5 mg/kg p.o.), after which dialysate was collected in 15-minute intervals for 2 hours (eight samples total). Microdialysis was performed in extracellular fluid as opioid receptors face the extracellular compartment; therefore, extracellular opioid concentrations are relevant to functional opioid response (Hammarlund-Udenaes et al, 2008).

Tissue collection, membrane preparation and ex vivo oxidation
Immediately following the microdialysis experiments, to confirm the impact of pre-treatments on brain CYP2D activity, with no impact of liver CYP2D activity and plasma oxycodone levels, animals were administered oxycodone and sacrificed at 30 minutes postinjection (approximately peak analgesia). Trunk blood was centrifuged at 5000 g for plasma isolation. Liver microsomal and brain cerebellum membranes were prepared as previously described (Miksys et al, 2017).
Formation of dextrorphan from dextromethorphan was assessed as a measure of \textit{ex vivo} CYP2D activity, as previously described (Miksys \textit{et al}, 2017; Tyndale \textit{et al}, 1999).

**Measurement of compounds**

Oxycodone and its metabolites (eight total compounds) were measured by modified published liquid chromatography–mass spectrometry methods (Andreassen \textit{et al}, 2011; Lalovic \textit{et al}, 2006); full methodological details can be found in the Supplementary Information.

**Statistical analyses**

Data were analyzed with GraphPad Prism v.6.01 (GraphPad Software Inc., La Jolla, CA, USA), by two-way ANOVA followed by either planned comparisons using one-way ANOVA (AUCs and CYP2D ratios), independent samples \(t\)-tests (AUCs) or Tukey’s \textit{post hoc} testing adjusted for multiple comparisons (%MPE at individual timepoints). Repeated measures were used in within-animal experiments. Relationships between data sets were assessed by Pearson correlation coefficients. Standard error of the mean (SEM) was displayed as unidirectional error bars for visual simplification.

4.4 Results

**Opioid analgesic dose–response was used to optimize oral oxycodone and oxymorphone doses**

Peak analgesia from a range of doses of oxycodone (5–15 mg/kg p.o.) and oxymorphone (2.5–10 mg/kg p.o.) was compared with that after various codeine and morphine doses and administration routes (Figure 25). The selected doses of oxycodone (12.5 mg/kg p.o.) and oxymorphone (10 mg/kg p.o.) (Figure 25, white circled) provided submaximal peak analgesic responses, allowing detection of both increases and decreases in analgesia, and compared with peak analgesia from doses of codeine (20 mg/kg i.p.) and morphine (3.5 mg/kg i.p.) used in a prior study assessing the impact of brain CYP2D on codeine response (Figure 25, black circled) (McMillan and Tyndale, 2015). Of note, a 4.3-fold lower oral oxycodone dose (used in this study) was required to produce similar peak analgesia relative to oral codeine (from Figure 25).
Modifying brain CYP2D activity resulted in altered oxycodone response

Compared with vehicle, propranolol (CYP2D inhibitor) i.c.v. pre-treatment increased %MPE at multiple timepoints (pre-treatment \( P = 0.044 \); Figure 26A) after oxycodone administration (12.5 mg/kg p.o.), resulting in a 1.9-fold increase of the analgesic area under the analgesic-time curve from 0 to 60 minutes (AUC\(_{0-60} \), \( P = 0.055 \); Figure 26B). A within-animal study design was used; animals received i.c.v. vehicle and i.c.v. propranolol in randomized order, with a 2-week washout between oxycodone (\( n = 15 \)). In addition, brain CYP2D was inhibited using a mechanistically (competitive versus mechanism-based inhibitor) and structurally distinct compound, propafenone. Compared with vehicle pre-treatment, propafenone pre-treatment increased %MPE at multiple timepoints (pre-treatment \( P = 0.003 \)) after oxycodone administration (12.5 mg/kg p.o.) resulting in a 3.7-fold increase of the analgesic AUC\(_{0-60} \) (\( P = 0.004 \); see Supplementary, Figure 31), providing further evidence that inhibiting brain CYP2D increases oxycodone analgesia (\( n = 7–8 \)).
Figure 26: Inhibiting brain CYP2D increased, and inducing brain CYP2D decreased, oxycodone analgesia. Compared with vehicle pre-treatment (V), propranolol pre-treatment (PL) resulted in significantly increased (a) analgesia (%MPE) and (b) area under the analgesia-time curve (AUC0-60) after oxycodone administration (12.5 mg/kg p.o., n = 15). Pre-treatment F(1, 14) = 4.89, P = 0.04. For AUC, t = 2.10, d.f. = 14, P = 0.05. Compared with vehicle pre-treatment, nicotine pre-treatment (NIC) resulted in significantly decreased (a) %MPE and (b) analgesic AUC0-60 after oxycodone administration (n = 17). Pre-treatment F(1, 16) = 12.76, P = 0.003. For AUC, t = 3.76, d.f. = 16, P = 0.002. Data are from two separate studies, and the averaged vehicle arm is shown; statistics are based on the individual experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle. %MPE, percentage of maximal possible effect; SEM, standard error of the mean.

Compared with vehicle, nicotine (CYP2D inducer) pre-treatment decreased %MPE at multiple timepoints (pre-treatment P = 0.003; Figure 26A) after oxycodone administration (12.5 mg/kg p.o.), resulting in a 1.6-fold decrease of the analgesic AUC0-60 (P = 0.002; Figure 26B). A within-animal study design was used; animals received 7-day s.c. vehicle and 7-day s.c. nicotine in randomized order, with a 2-week washout between oxycodone (n = 17). These data together suggest that the parent compound oxycodone, rather than the more potent metabolite oxymorphone, is responsible for oxycodone analgesia.
Modifying brain CYP2D alters oxycodone, but not oxymorphone, analgesia

The impact of modifying brain CYP2D activity on oxycodone analgesia was replicated (Figures 26 and 27A); compared with vehicle pre-treatment, inhibiting brain CYP2D with propranolol resulted in higher %MPE and analgesic AUC$_{0-60}$ ($P < 0.01$), while inducing brain CYP2D with nicotine resulted in a lower %MPE at 30 minutes, and lower analgesic AUC$_{0-60}$ ($P < 0.001$) compared with propranolol pre-treatment, after oxycodone administration (12.5 mg/kg p.o.). Inhibiting the nicotine pre-treatment-induced brain CYP2D with i.c.v. propranolol yielded no difference in %MPE or analgesic AUC$_{0-60}$ ($P = 0.47$; Figure 27A) after oxycodone administration, compared with vehicle ($n = 10–13$). Inhibition of the nicotine pre-treatment effect on oxycodone analgesia suggests that propranolol and nicotine pre-treatment impact analgesia through a common mechanism, i.e. modification of brain CYP2D activity.

Oxymorphone is the CYP2D metabolite of oxycodone and is not further metabolized by CYP2D; pre-treatments that selectively inhibit or induce brain CYP2D should therefore not affect oxymorphone analgesia. Compared with vehicle pre-treatment, propranolol pre-treatment, nicotine pre-treatment and combined pre-treatment of nicotine and propranolol did not alter %MPE (at any timepoints) or AUC$_{0-60}$ ($P > 0.6$ for all pre-treatment groups; Figure 27B) after oxymorphone administration (10 mg/kg p.o., $n = 10–11$). This suggests that the effect of pre-treatments (propranolol and/or nicotine) on oxycodone analgesia was likely due to altered central CYP2D-mediated metabolism of oxycodone rather than alternative analgesic or kinetic effects (e.g. on BBB transport or opioid-receptor activation).

Baseline TFLs (in seconds) were assessed prior to experimentation, and there were no significant differences across pre-treatment groups just prior to oxycodone ($P = 0.21$; vehicle $= 3.6 + 0.1$, mean $\pm$ SEM, propranolol $= 3.2 + 0.2$, nicotine $= 3.2 + 0.1$ and nicotine and propranolol $= 3.4 + 0.2$) or oxymorphone ($P = 0.88$; vehicle $= 4.5 + 0.4$, propranolol $= 4.3 + 0.3$, nicotine $= 4.7 + 0.4$ and nicotine and propranolol $= 4.7 + 0.2$) administration, as previously observed (McMillan and Tyndale, 2015).
Figure 27: Inhibiting induced brain CYP2D reversed the changes in oxycodone analgesia; variation in brain CYP2D activity did not affect oxymorphone analgesia. Compared with vehicle pre-treatment (V), propranolol pre-treatment (PL) or nicotine pre-treatment (NIC) alone resulted in significantly higher and lower, respectively, (a) %MPE and analgesic AUC$_{0-60}$ after oxycodone administration (12.5 mg/kg p.o., $n = 10–13$ group). Compared with vehicle, combined nicotine and propranolol pre-treatment (NICPL) resulted in a significantly different %MPE and AUC$_{0-60}$ than nicotine or propranolol pre-treatment alone and no change in %MPE or AUC$_{0-60}$ from vehicle pre-treatment. Pre-treatment $F(3, 42) = 6.90$, $P = 0.001$. For AUCs, $F(3, 42) = 7.53$, $P < 0.001$. Compared with vehicle pre-treatment, a higher portion of animals pre-treated with propranolol reached maximal analgesia, while no animals pre-treated with nicotine reached maximal analgesia (a, table). Compared with vehicle pre-treatment, propranolol pre-treatment, nicotine pre-treatment and co-pre-treatment of nicotine and propranolol did not result in significantly different (b) %MPE or AUC$_{0-60}$ after oxymorphone administration (10 mg/kg p.o., $n = 10–11$ group). For analgesia curves, pre-treatment $F(3, 34) = 0.21$, $P = 0.89$. For AUCs, $F(3, 34) = 0.14$, $P = 0.94$. A similar percentage of animals reached maximal oxymorphone analgesia across all pre-treatment groups (b, table). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with vehicle and #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with nicotine and propranolol combined pre-treatment. %MPE, percentage of maximal possible effect; SEM, standard error of the mean.
Brain oxycodone, not oxymorphone, levels are responsible for oxycodone analgesia

To investigate how the differences in oxycodone analgesia were related to changes in brain oxycodone or oxymorphone levels in vivo, microdialysis was used to assess brain analyte levels over time after oxycodone. After oxycodone administration (12.5 mg/kg p.o.) in vehicle pre-treated animals, oxycodone and noroxycodone were the most abundant analytes, followed by noroxymorphone and oxymorphone-glucuronide, and oxymorphone was the least abundant (Figure 28A; curves for all analytes in the pre-treatment arms are in Supplementary Information, Figure 32).

Compared with vehicle, propranolol pre-treatment significantly increased brain oxycodone levels ($P < 0.05$; Figure 28C) after oxycodone administration, consistent with an increase in oxycodone analgesia (Figure 28B). Nicotine pre-treatment increased brain oxymorphone levels ($P < 0.001$; Figure 28G); this was not consistent with the decrease in oxycodone analgesia. Together these brain analyte and time course data provide evidence to suggest that oxycodone, rather than the more potent CYP2D metabolite oxymorphone, is responsible for oxycodone analgesia. Combined nicotine and propranolol pre-treatment blocked the effect of propranolol on brain oxycodone levels ($P = 0.12$; Figure 28I) and nicotine on brain oxymorphone levels ($P = 0.56$; Figure 28J), consistent with the oxycodone analgesia data (Figure 28H).
Figure 28: Manipulating brain CYP2D further suggests that brain oxycodone levels, not oxymorphone levels, are responsible for oxycodone analgesia. Using in vivo brain microdialysis, oxycodone (OC) and four other metabolites [noroxycodone (NOC), oxymorphone (OM, on secondary axis), noroxymorphone (NOM) and oxymorphone-glucuronide (OMG)] could be detected after oxycodone administration (12.5 mg/kg p.o., vehicle arm in a). Compared with vehicle pre-treatment (V), propranolol pre-treatment (PL) increased (b) analgesia (from Figure 27A) and (c) brain oxycodone levels ($F(1, 15) = 5.20, P = 0.03$), but had no effect on (d) brain oxymorphone levels ($F(1, 15) = 0.28, P = 0.60$), after oxycodone administration. Nicotine pre-treatment (NIC) decreased (e) analgesia (from Figure 27A) and increased (g) brain oxymorphone levels ($F(1, 13) = 15.85, P < 0.001$) but had no effect on (f) brain oxycodone levels ($F(1, 13) = 1.52, P = 0.22$), after oxycodone administration. Combined nicotine and propranolol pre-treatment (NICPL) did not affect (h) analgesia (from Figure 27A), (i) brain oxycodone levels ($F(1, 15) = 2.54, P = 0.12$) or (j) brain oxymorphone levels ($F(1, 15) = 0.34, P = 0.56$) after oxycodone administration ($n = 5–9$/group). *$P < 0.05$, ***$P < 0.001$. %MPE, percentage of maximal possible effect; SEM, standard error of the mean.
Manipulating brain CYP2D altered brain, but not liver, oxycodone metabolism: oxycodone analgesia correlated with brain CYP2D activity

Brain CYP2D metabolic ratios (metabolite oxymorphone level/substrate, oxycodone level; n = 5/9) from microdialysis data and plasma CYP2D metabolic ratios at 30 minutes post-oxycodone (12.5 mg/kg p.o., n = 12) were calculated for each pre-treatment group. The overall brain CYP2D metabolic ratio AUC₀–₆₀ (encompassing peak analgesia) was greater in nicotine pre-treated animals (P < 0.05 against all groups) and different across pre-treatment groups (F(3, 24) = 4.34, P = 0.014). The brain CYP2D metabolic ratio from the microdialysis collection time of 15–30 minutes was illustrated (Figure 29A) to contrast most closely with the plasma CYP2D metabolic ratio from the plasma sampling time of 30 minutes. Compared with vehicle, propranolol pre-treatment decreased the brain CYP2D metabolic ratio (n = 5–9; Figure 29A), but did not alter the plasma CYP2D metabolic ratio (Figure 29C), and nicotine pre-treatment increased the brain CYP2D metabolic ratio (P < 0.05 against all groups; Figure 29A), but did not alter the plasma CYP2D metabolic ratio (Figure 29C). Combined nicotine and propranolol pre-treatment blocked the effects of nicotine or propranolol pre-treatment alone, evidenced through no change in brain or plasma CYP2D metabolic ratios (Figures 29A and C). Compared with vehicle (7.82 ± 0.38 nmol/g/h, mean ± SEM), propranolol pre-treatment modestly decreased (7.01 ± 0.35 nmol/g/h), nicotine pre-treatment modestly increased (8.74 ± 0.55 nmol/g/h) and combined nicotine and propranolol pre-treatment resulted in no change in (7.91 ± 0.55 nmol/g/h) brain CYP2D activity (F(3, 82) = 1.37, P = 0.26). Hepatic CYP2D activity was unchanged across all four pre-treatment groups (F(3, 44) = 0.02, P = 0.99). Analgesia (at 30 minutes, approximately peak) negatively correlated with ex vivo brain CYP2D activity (n = 20–23; P = 0.002; Figure 29B), but not with hepatic CYP2D activity (n = 12; P = 0.42; Figure 29D). These findings further indicate that manipulations of brain CYP2D metabolism alter brain, but not hepatic, oxycodone metabolism and resulting analgesia.
Figure 29: Manipulating brain CYP2D altered the CYP2D metabolic ratio in the brain; oxycodone analgesia was inversely correlated with brain CYP2D activity. (a) Compared with vehicle pre-treatment (V), the brain CYP2D metabolic ratio (oxymorphone/oxycodone levels) was slightly lower in animals given propranolol pre-treatment (PL), higher in animals given nicotine pre-treatment (NIC) and unaltered in the combined propranolol and nicotine (NICPL) pre-treatment group after oxycodone administration (12.5 mg/kg p.o., n = 5–9/group; F(3, 24) = 0.96, P = 0.43). (b) Oxycodone analgesia was significantly and inversely correlated with brain CYP2D activity (P < 0.002) at 30 minutes (approximately peak) after oxycodone administration (n = 20–23/group). (c) The plasma CYP2D metabolic ratio was similar across all pre-treatment groups (n = 12/group; F(3, 67) = 0.037, P = 0.99). (d) Oxycodone analgesia was not correlated with hepatic CYP2D activity (P = 0.42) at 30 minutes after oxycodone administration (n = 12/group). %MPE, percentage of maximal possible effect; SEM, standard error of the mean.

4.5 Discussion
This study demonstrated an impact of brain CYP2D on central oxycodone metabolism to oxymorphone and resulting analgesia. Inhibiting rat brain CYP2D increased oxycodone levels and analgesia, while inducing rat brain CYP2D increased oxymorphone levels and decreased analgesia; inhibiting the induced brain CYP2D blocked the decrease in oxycodone analgesia. Together, this demonstrates that variation in brain CYP2D alters oxycodone brain levels and...
response. Pre-treatments modestly altered CYP2D metabolic ratios in brain, but not in plasma; oxycodone analgesia was inversely correlated with \textit{ex vivo} brain, but not liver, CYP2D activity. Additionally, pre-treatments altering brain CYP2D activity did not affect baseline TFLs or oxymorphone analgesia, indicating that the pre-treatments had no effect on (1) nociception, (2) pathways mediating antinociception, (3) transport across the BBB and (4) peripheral CYP2D activity. These findings provide strong evidence that brain oxycodone, rather than the more potent \(\mu\)-opioid receptor activating metabolite oxymorphone, is the primary contributor to oxycodone analgesia and that levels of brain CYP2D influence oxycodone levels and response.

The role of brain CYP2D metabolism in oxycodone analgesia directly contrasts with its role in codeine and tramadol analgesia. Like oxycodone, codeine and tramadol are metabolized by CYP2D to more potent opioid-receptor agonists morphine and \(O\)-desmethyltramadol, respectively (Chen \textit{et al}, 1991b; Grond and Sablotzki, 2004). However, codeine and tramadol analgesia were increased as a result of brain CYP2D metabolism increasing brain levels of the CYP2D metabolites; codeine analgesia correlated with brain morphine levels, while tramadol analgesia correlated with CSF \(O\)-desmethyltramadol levels (McMillan and Tyndale, 2015; Wang \textit{et al}, 2015). Thus, brain CYP2D has opposing impacts on analgesia from different opioids; higher brain CYP2D lowers oxycodone analgesia, likely through reduced brain oxycodone levels, but increases codeine and tramadol analgesia, likely through higher levels of the active metabolites.

Despite an established role for hepatic CYP2D in metabolism of oxycodone, both pre-clinical and human studies show little evidence for a role of hepatic CYP2D metabolism in oxycodone analgesia. Inhibiting rat CYP2D (quinine) \textit{in vivo} did not alter oxycodone analgesia (Cleary \textit{et al}, 1994). Likewise, while inhibiting human CYP2D6 \textit{in vivo} (quinidine or paroxetine) reduced plasma oxymorphone AUC\(_{0-48h}\) (40 percent) and increased plasma noroxycodone AUC\(_{0-48h}\) (30 percent), it did not alter oxycodone plasma concentrations or analgesia (Gronlund \textit{et al}, 2010). Furthermore, inhibiting human CYP2D6 \textit{in vivo} does not impact the analgesic or reinforcing effects of other structurally related 6-keto-opioids hydrocodone and dihydrocodeine (Kaplan \textit{et al}, 1997; Lelas \textit{et al}, 1999; Schmidt \textit{et al}, 2003; Webb \textit{et al}, 2001). The finding here that brain oxycodone, despite being a less potent \(\mu\)-opioid receptor activator than oxymorphone, is primarily responsible for analgesia is counterintuitive; the expectation being greater formation of the more
potent oxymorphone should have increased analgesia. Oxycodone is a relatively potent μ-opioid receptor agonist and readily crosses the BBB into the CNS, whereas oxymorphone has low systemic levels and BBB permeability (1.6-fold lower brain-to-plasma ratio in rats) (Bostrom et al, 2006; Klimas et al, 2013; Lalovic et al, 2006; Riley et al, 2008; Sadiq et al, 2013). The differences in brain-to-plasma partitioning, sequestration, relative amounts and clearance between oxycodone and oxymorphone may be sufficient to prevent changes in brain oxymorphone levels from influencing oxycodone analgesia under these conditions (i.e. increased oxymorphone levels when brain CYP2D is induced).

Our nicotine pre-treatment paradigm in rats, which induces brain CYP2D, results in average daily nicotine plasma levels (25 ng/ml) comparable with smokers (20–50 ng/ml); smoking is associated with elevated human brain CYP2D (Benowitz, 1999; Mann et al, 2008; Miksys et al, 2002; Yue et al, 2008). Non-medicinal prescription opioid use is higher in smokers than non-smokers; however, the isolated effects of smoking on acute oxycodone response are unclear (Skurtveit et al, 2010). In a study assessing the influence of smoking status on oxycodone response, smokers experienced less oxycodone-induced nausea (Zacny et al, 2013), which is a common opioid-receptor-mediated side effect of oxycodone treatment (He et al, 2016). Increased brain CYP2D activity in smokers, increasing central metabolism of oxycodone and potentially reducing μ-opioid receptor occupancy, could contribute to the decreased oxycodone-induced nausea experienced in smokers. It is possible that decreases in opioid response and/or nausea alter the opioid-taking experience and abuse potential of oxycodone among smokers.

The effect of smoking on oxycodone abuse liability is currently unknown. Theoretically, it is difficult to discern whether inducing brain CYP2D might decrease the abuse potential of oxycodone due to decreased acute effect or potentially increase the abuse potential due to decreased adverse side effects. In a study assessing the reinforcing effects of oxycodone, 67 percent of the oxycodone drug abusers were smokers compared with 22 percent of the non-abusers (Comer et al, 2010). Cigarette smoking is strongly associated with illicit drug use (Eckhardt et al, 1994); separating the effect of smoking on brain reward systems and on inducing brain CYP2D requires alternative methods. Numerous pharmacological characteristics have been linked to opioid abuse potential, such as onset and duration of action (Wightman et al, 2012). In this study,
nicotine pre-treatment modestly delayed time to peak analgesia (30.5 ± 1.30 minutes, mean ± SEM) compared with all other pre-treatments (vehicle: 27.5 ± 1.7 minutes; propranolol: 24.2 ± 1.8 minutes; and nicotine and propranolol: 26.8 ± 1.4 minutes; \( F(3, 42) = 1.66, P = 0.19 \)). Thus, the subjective effects of a decreased and delayed peak oxycodone effect on altering the rewarding properties and the transition to abuse remain to be directly investigated.

Oxycodone and metabolite levels were assessed by microdialysis in the cerebral ventricles of a subset of animals and compared with levels in dialysate from the nucleus accumbens. There was no difference in rank order of oxycodone and metabolites at any timepoint \( (P > 0.05) \) and no difference in metabolite levels across (ANOVA of brain CYP2D ratio: \( F(1,9) = 0.016, P = 0.90 \)), suggesting drug levels in the nucleus accumbens shell and the cerebral ventricles were similar. Therefore, the nucleus accumbens was used to enhance our ability in the future to pair brain opioid levels with opioid-induced dopamine release. Only male rats were used in this study as previous studies optimizing the pre-treatment paradigms were performed in male rats; exploration of male–female sex differences in brain CYP2D activity and functional influences is currently being planned. We used one behavioral assay for antinociception (tail flick) because of its reliability and reproducibility (Grossman et al., 1982); future studies expanding these findings with additional antinociception assays (e.g. hot plate), as well as additional outcomes of human clinical oxycodone use (e.g. opioid-induced side effects, tolerance and dependence), would assist in understanding the extent of the impact of central oxycodone metabolism.

This study provides further evidence of an impact of rat brain CYP2D on opioid metabolism and response. Increasing (or decreasing) brain CYP2D activity and the consequent increase (or decrease) in oxycodone metabolism decreased (or increased) oxycodone analgesia. Together with microdialysis assessments, the results suggest that oxycodone is the active analgesic moiety in this paradigm. Individuals with increased brain CYP2D activity (e.g. smokers and genetically rapid CYP2D metabolizers) may experience lower oxycodone analgesia, while those with lower brain CYP2D activity (e.g. non-smokers or those taking centrally acting CYP2D inhibitors) may experience greater analgesia. This work also illustrates a contrasting role for brain CYP2D in oxycodone versus codeine and tramadol analgesia. Hormones (e.g. testosterone and estrogen) and developmental factors (e.g. age) can also influence brain CYP2D activity (Baum and Strobel,
1997; Mann et al, 2012); the combined impact of genetic, environmental and hormonal factors on brain CYP activity and on clinically relevant outcomes of altered CNS drug metabolism is still a relatively unknown source of inter-individual variation in drug response. Investigation into potential sources of individual variation in opioid response remains essential for identifying risk factors for altered analgesic response and opioid abuse.

4.6 Acknowledgements
We would like to thank Dr. Bin Zhao for his invaluable technical assistance with the liquid chromatography–mass spectrometry assay and Fariba Baghai Wadji for her expert support with microdialysis animal procedures. This work was supported by a Canada Research Chair in Pharmacogenomics; the Canadian Institutes of Health Research (grant numbers FDN 154294, TMD 132557 and MOP 136937); the Campbell Family Mental Health Research Institute of the Centre for Addiction and Mental Health (CAMH) and the CAMH Foundation; the Canada Foundation for Innovation (grant numbers 20289 and 16014); and the Ontario Ministry of Research and Innovation. Dr. Rachel F. Tyndale has consulted for Apotex on topics unrelated to this work. Dr. Sharon Miksys and Douglas M. McMillan have no conflicts of interest to declare.

4.7 Supplementary Information
**LC-MS quantification of oxycodone and analyte levels in biological samples** (adapted from (Andreassen et al, 2011; Lalovic et al, 2006))

**Abbreviations**
Oxycodone (OC), noroxycodone (NOC), oxymorphone (OM), noroxymorphone (NOM), 6-α-oxycodol (OCOL), 6-α-oxymorphol (α-OMOL), 6-β-oxymorphol (β-OMOL), oxycodone N-oxide (OCO) and oxymorphone-3-β-D-glucuronide (OMG) and their internal standards 6-α-oxycodol-d₃ (OCOL-d₃), and noroxycodone-d₃ (NOC-d₃) were purchased from Toronto Research Chemicals.
**Instrumentation**

*HPLC System:*

HPLC system (Agilent 1260 LC system): Agilent 1260 Quaternary pump, Agilent 1260 Infinity Standard Autosampler and temperature-controlled column compartment. OC, NOC, OM, NOM, OCOL, α-OMOL, β-OMOL, OCO, OMG, OCOL-d₃ and NOC-d₃ were separated on an Agilent ZORBAX SB-C18 column (15 cm x 2.1 mm I.D.; 5 µm particle size; Agilent Technologies, Palo Alto, CA) at ambient temperature with a flow rate of 0.3 ml/min.

### HPLC gradient:

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*Mass spectrometry:*

Instrument: Agilent 6430 Triple Quadrupole LC/MS system

Software: MassHunter software.

Acquisition Mode: multiple reaction monitoring (MRM)

Ionization: electrospray ionization (ESI)

Gas temperature: 350° C

Gas flow: 10 l/min

Nebulizer pressure: 35 psi

Capillary voltage: 3000 V
**Internal standards:**

Working solution 20 ng/ml of OCOL-d3 and NOC-d3 prepared in water.

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</tr>
<tr>
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<td>17</td>
<td>10.6</td>
<td>OCOL-d3</td>
</tr>
<tr>
<td>α-OMOL</td>
<td>304.1 → 286.1</td>
<td>21</td>
<td>3.2</td>
<td>OCOL-d3</td>
</tr>
<tr>
<td>β-OMOL</td>
<td>304.1 → 286.1</td>
<td>17</td>
<td>4.5</td>
<td>OCOL-d3</td>
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<td>332.1 → 315.1</td>
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<tr>
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<td>29</td>
<td>1.9</td>
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<tr>
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<td>17</td>
<td>10.3</td>
<td>-</td>
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<tr>
<td>NOC-d3</td>
<td>305.2 → 287.1</td>
<td>13</td>
<td>14.3</td>
<td>-</td>
</tr>
</tbody>
</table>

**Sample Preparation**

**Microdialysis**

Dialysis samples (30 µl) were mixed with internal standards (10 µl of 20 ng/ml deuterated metabolites in dH2O). Samples were centrifuged at 12,000 rpm for 10 min, then 40 µl was injected into the LCMS system. LOQ = 0.1ng/ml for OC, NOC, OM, NOM, OCOL, α-OMOL, β-OMOL, OCO and OMG (30ul dialysis sample used). The standard curve was calibrated with linear function and 1/x weighing. Coefficient of correlation should be > 0.99. The calibration curve was prepared in the same biological matrix as the samples (Ringer’s solution) with known concentrations from a working solution of all analytes assessed; calibration curve range: 0.1, 0.2, 0.5, 1, and 10 ng/ml.

**Plasma and Brain Homogenate**

Internal standards (20 µl of 20 ng/ml deuterated metabolites in dH2O) were added to 100 µl of sample, and protein was precipitated with acetonitrile (0.9 ml). Samples were vortexed for 30 seconds, centrifuged at 12,000 rpm for 10 min, the supernatant evaporated to dryness under nitrogen at 38°C, reconstituted with 100 µl of mobile phase (ammonium acetate buffer /
acetonitrile, 95 / 5 %), and injected (60 µl) into the LCMS system. LOQ: 0.2ng/ml (100ul used) for OC, NOC, OM, OCO, OMG, OCOL, 6-alpha-OMOL, 6-beta-OMOL; 0.5ng/ml for NOM (100ul used). The standard curve was calibrated with linear function and 1/x weighing. Coefficient of correlation should be > 0.99. The calibration curve was prepared in the same biological matrix as the samples (control plasma) with known concentrations from a working solution of all analytes assessed; calibration curve range: 0.2, 0.5, 1, 10, 100, and 500 ng/ml.

**Figure 30:** Study paradigm of pretreatment timing prior to oxycodone or oxymorphone administration. Nicotine pretreatment is given subcutaneously (1 mg/kg, s.c.) once daily for seven days; oxycodone or oxymorphone are given at 8 hr after the last nicotine pretreatment. Propranolol pretreatment is by a single intracerebroventricular injection (20 µg in 4ul cyclodextrin, i.c.v.) 24 hr prior to oxycodone or oxymorphone administration.
Figure 31: Inhibiting brain CYP2D with the competitive inhibitor propafenone increased oxycodone analgesia. Compared with vehicle pretreatment, propafenone pretreatment (brain CYP2D inhibitor, 30 min prior) resulted in significantly increased %MPE (A; F (1, 13) = 13.32, p = 0.003) and AUC$_{0-60 \text{ min}}$ (B; un-paired t = 3.51, df = 13, p = 0.004) after oxycodone administration (12.5 mg/kg p.o., n=7-8/group). The weighted average of the vehicle groups (including vehicle groups from Figure 26) was calculated and shown for illustration of the comparisons with those in Figure 26; statistics shown here are based on this individual experiment. **p<0.01, ***p<0.001.
Figure 32: Full microdialysis analyte curves for each pretreatment group after oxycodone administration. Drug levels by time are displayed for vehicle pretreatment (A), propranolol pretreatment (B), nicotine pretreatment (C) and combined nicotine and propranolol pretreatment (D). Oxycodone (OC) and its three primary metabolites noroxycodone (NOC), oxymorphone (OM, on secondary axis), and noroxymorphone (NOM), along with the additional metabolite oxymorphone-glucuronide (OMG) could be detected in all four pretreatment groups after oxycodone administration (12.5 mg/kg, p.o., n=5-9/group).
4.8 Significance to Thesis

This study provides further evidence of an impact of brain CYP2D on opioid metabolism and response, building from the prior two studies by using a distinct opioid compound oxycodone. Inhibiting rat brain CYP2D increased analgesia, while inducing rat brain CYP2D decreased analgesia after oral oxycodone administration. Furthermore, using *in vivo* brain microdialysis optimized to detect brain levels of oxycodone and its primary metabolites over time, we found that inhibiting brain CYP2D increased brain oxycodone levels while inducing brain CYP2D increased brain oxymorphone (CYP2D-metabolite of oxycodone) levels *in vivo*. While oxycodone, like codeine, is metabolized by CYP2D to a more potent opioid receptor-activating metabolite oxymorphone, our *in vivo* brain microdialysis data indicated that the parent compound oxycodone is predominantly responsible for oxycodone-induced analgesia. This is in contrast to our findings on the impact of brain CYP2D metabolism on codeine response, where the CYP2D-metabolite morphine was primarily responsible for codeine-induced analgesia, suggesting different roles for brain CYP2D in response to these two opioids.

Findings from this chapter suggest that individuals with high brain CYP2D activity (e.g. smokers, individuals taking nicotine) may exhibit a lower response to oxycodone due to increased central metabolism of oxycodone to oxymorphone. Human studies have found no contribution of *CYP2D6* genotype to overall oxycodone effect, therefore this study provides a potential novel source of interindividual variation in oxycodone response. The findings outlined in this chapter support future work on elucidating the impact of brain CYP2D metabolism on additional substrate opioid compounds such as hydrocodone.
5. General Discussion

5.1 Summary of findings

5.1.1 Brain CYP2D and codeine

The studies in this thesis demonstrated a novel impact of brain CYP2D on the central metabolism of opioids and resulting analgesia in vivo, after acute and repeated opioid administration. In chapter 1, rats were administered intraperitoneal doses of codeine and morphine adjusted to produce similar plasma morphine levels at 15 min post-injection, the time of peak codeine analgesia; animals administered codeine had higher brain morphine levels and analgesia than those administered morphine, consistent with brain CYP2D metabolism of codeine. We showed that inhibiting CYP2D specifically in the brain with direct intracerebroventricular injection of mechanism-based, and competitive, CYP2D inhibitors decreased brain morphine levels and analgesia after intraperitoneal codeine administration. We further showed that inducing brain CYP2D with short-term nicotine pretreatment increased brain morphine levels and analgesia after codeine, which was reversed by inhibiting brain CYP2D. Inhibiting and inducing brain CYP2D did not alter morphine-induced analgesia, indicating the selectivity of pretreatment effect on codeine analgesia was through brain CYP2D alone. Codeine analgesia correlated with brain, but not plasma, morphine levels, consistent with brain CYP2D-mediated activation of codeine to morphine primarily mediating codeine analgesic response (Figure 33).

In chapter 2, we investigated the role of brain CYP2D metabolism in response to chronic drug administration. Specifically, we assessed whether inducing brain CYP2D, and thereby increasing the brain CYP2D activation of codeine to morphine, would impact the rate of onset of codeine analgesic tolerance during daily oral codeine administration. We found that inducing brain CYP2D with short-term nicotine pretreatment increased acute codeine analgesia (replicating our findings in chapter 1) and the rate of codeine tolerance, quantified as a greater decrease (greater slope) in peak analgesia (30 minutes post-injection) across 7 days of oral codeine administration. Consistent with morphine not being a CYP2D substrate, inducing brain CYP2D did not alter the rate of tolerance to analgesia from daily oral morphine administration. The rate of tolerance to codeine correlated with acute analgesia, which was increased by inducing brain CYP2D, while the rate of tolerance to morphine also correlated with acute analgesia, which was unaffected by brain CYP2D.
The same relationship was seen when acute analgesia was increased by administering higher doses of codeine and morphine, further indicating acute analgesia was predominantly responsible for the rate of tolerance. Individuals who experience a greater acute codeine analgesic effect, either by taking a high codeine dose or by having higher brain CYP2D activity (e.g. due to genetics or exposure to xenobiotic inducers), may experience a greater absolute decrease, but similar proportional decrease, in codeine analgesia across repeated doses, than those with a lower acute analgesic effect. The potential clinical outcome of this phenomenon is unknown, but is explored further in discussion section 5.3.

![Proposed Model of Codeine-Induced Analgesia](image)

**Figure 33: Proposed model for the functional role of rat brain CYP2D in the activation of codeine to morphine in vivo.** A) While codeine readily enters the CNS, morphine has difficulty crossing the blood-brain barrier (BBB) and is actively effluxed. B) Inhibiting CYP2D specifically in the brain with the mechanism-based inhibitor propranolol (PL, metabolized by CYP2D to hydroxy-propranolol [OH-PL] which binds irreversibly to the active site of CYP2D rendering it inactive) decreased brain morphine formation from codeine, and resulting codeine analgesia. C) In contrast, inducing brain CYP2D with short-term nicotine (NIC) pretreatment increased brain morphine formation from codeine and resulting codeine analgesia.

5.1.2 Brain CYP2D and oxycodone

In chapter 3, we extended our findings on brain CYP2D metabolism to oxycodone, an opioid compound with a pharmacologic profile distinct from codeine. We were initially unsure if greater brain CYP2D metabolism of oxycodone would increase oxycodone analgesic response as, like codeine, oxycodone is metabolized by CYP2D to a more potent MOR-activating compound, oxymorphone. However, inhibiting brain CYP2D with propranolol increased analgesia and brain
oxycodone levels, measured by microdialysis after oral oxycodone administration, suggesting that the parent compound was responsible for oxycodone analgesia. Inducing brain CYP2D with short-term nicotine pretreatment increased brain oxymorphone levels from oral oxycodone, but decreased analgesia, consistent with brain oxymorphone levels not contributing to oxycodone analgesia under this paradigm. The effect of inducing brain CYP2D on oxycodone analgesia and brain oxymorphone levels was blocked by inhibiting brain CYP2D. Similar to the morphine control used in chapters 1 and 2, we found that oxymorphone analgesia (not a CYP2D substrate) was not altered by the pretreatments used to increase and decrease brain CYP2D activity, suggesting the effects of pretreatment on oxycodone analgesia and drug levels were due to changes in brain CYP2D activity alone. Oxycodone analgesia negatively correlated with ex vivo brain, but not liver, CYP2D activity consistent with oxycodone being primarily responsible for oxycodone analgesia. Due to BBB permeability differences between oxycodone and oxymorphone, the brain CYP2D metabolism of oxycodone may function to increase clearance of oxycodone from the CNS (Figure 34); this concept is further explored in discussion section 5.2.

**Figure 34:** Proposed model for the functional role of rat brain CYP2D in the metabolism of oxycodone to oxymorphone in vivo. A) Oxycodone partitions from plasma across the blood-brain barrier (BBB) into the CNS, while oxymorphone is produced in low quantities peripherally and centrally. B) Inhibiting CYP2D specifically in the brain with the mechanism-based inhibitor propranolol (PL, metabolized by CYP2D to hydroxy-propranolol [OH-PL] which binds irreversibly to the active site of CYP2D rendering it inactive) increased brain oxycodone levels, and resulting oxycodone analgesia. C) In contrast, inducing brain CYP2D with nicotine (NIC) increased brain oxymorphone from oxycodone, and decreased oxycodone analgesia.
5.2 Predicting the contribution of opioid parent and CYP2D metabolite to drug effect

5.2.1 Pharmacokinetics, pharmacodynamics and blood-brain barrier transport

The studies in this thesis have focused on elucidating the relative contribution of parent (codeine and oxycodone) and brain CYP2D-mediated metabolite (morphine and oxymorphone) to opioid drug effect (analgesia and tolerance). This is predominantly dependent on the pharmacokinetic and pharmacodynamic properties of each compound, including the proportion of parent opioid compound metabolized by CYP2D, the metabolite production in the periphery (primarily liver) relative to the brain, the influx across the BBB relative to efflux from the CNS, and target receptor affinity (Smith, 2009, 2011). These variables differ between opioid compounds, and account for differences in the impact of brain CYP2D metabolism on opioid effect, such as that seen in chapters 1 and 3.

5.2.1.1 Codeine

The relative contribution of codeine and the CYP2D-mediated metabolite morphine to codeine-induced analgesia is fairly straightforward. Morphine is a relatively minor metabolite of codeine, representing 0–15% of a codeine dose, while 80% of codeine is metabolized by UGT2B7 to codeine-6-glucuronide, and the remainder by CYP3A4 to norcodeine (Chen et al., 1991b; Smith, 2011). The affinity of morphine for the MOR is approximately 3000-fold greater than codeine and morphine is therefore primarily responsible for codeine analgesia (Smith, 2011). There are established differences in the amount of morphine produced from codeine and resulting codeine analgesia between individuals given CYP2D6 inhibitors (e.g. quinidine) and vehicle, and between individuals with different CYP2D6 genotypes (Sindrup et al., 1992; Sindrup et al., 1990). Our findings in chapter 1 suggest that while morphine formed from codeine is responsible for codeine analgesia, distinct properties of the two compounds, like BBB permeability, may reduce the contribution of peripherally formed morphine (via hepatic CYP2D) to analgesia, which is a centrally mediated effect.

There are marked differences in the ability of codeine and morphine to cross the BBB. This was shown using in vivo brain microdialysis in rats infused intravenously with codeine or morphine, where codeine rapidly entered into brain extracellular fluid, and exhibited identical influx and
efflux clearances, producing an unbound brain-to-blood AUC ratio of 1.00 ± 0.18 (Xie and Hammarlund-Udenaes, 1998). In comparison, morphine produced a 3.6-fold lower brain-to-blood AUC ratio (0.28 ± 0.09) than codeine, and a net efflux clearance (26 µl/min*g-brain), indicating both poor BBB permeability and active efflux (Bouw et al, 2000; Xie and Hammarlund-Udenaes, 1998). Consistent with this, in chapter 1, there was a correlation between plasma and brain codeine, but not morphine, levels at 15 min post-codeine injection, indicating that codeine, but not morphine, readily equilibrates across the BBB.

Comparing compartment-specific half-lives (t\(1/2\)) in rats infused with codeine or morphine, codeine displayed a similar t\(1/2\) in brain (26 min) and blood (24 min), while morphine had a significantly longer t\(1/2\) in brain (44 min) than in blood (30 min) suggesting potential redistribution within brain tissue (Bouw et al, 2000). In chapter 1, when rats were given intraperitoneal doses of codeine (20 mg/kg) or morphine (3.5 mg/kg), producing similar plasma morphine levels at 15 min post-injection, codeine analgesia peaked at approximately 15 min and dropped below 50% (MPE) by 30 min, while morphine analgesia peaked at 30 min post-injection and dropped below 50% at 60 min. The differences in compartment-specific half-lives, along with the slow BBB influx into the CNS, could account for the delay in onset and greater duration of morphine analgesia compared to codeine. Rats given codeine had approximately 6-fold greater brain morphine levels at the time of peak codeine analgesia than rats given morphine. This suggests that central morphine levels and analgesia at this time were likely derived from codeine (rapidly influxed) via central CYP2D-mediated activation to morphine, rather than from peripheral morphine (slowly influxed) produced via hepatic CYP2D.

Furthermore, efflux transporters at the BBB (e.g. P-glycoprotein [P-gp]) can function as determinants of the bioavailability of opioids like codeine and morphine (Thompson et al, 2000; Wandel et al, 2002). Codeine and morphine are both substrates of P-gp, evidenced by their ability to increase P-gp mediated ATP hydrolysis in vitro (Cunningham et al, 2008). However, due to the ability of codeine to passively permeate across the BBB, P-gp mediated active transport has no effect on its distribution across the BBB (Cunningham et al, 2008). P-gp mediated efflux of morphine is important in reducing morphine analgesic effect; P-gp knockout mice had greater analgesia from morphine, and individuals homozygous for a variant SNP in the gene encoding P-
gp (ATP-binding cassette B1 gene) had greater pain relief from morphine (Campa et al., 2008; Thompson et al., 2000). Differences in P-gp efflux at the BBB may contribute to differences in efflux of codeine and morphine from the CNS, and provide additional understanding for the findings in chapters 1 and 2. The low BBB permeability of peripherally-formed morphine, as well as active efflux via P-gp, suggest that morphine present in the brain shortly after codeine administration is due to central metabolism of codeine. This explains, in part, why inhibiting and/or inducing the brain CYP2D activation of codeine to morphine altered brain morphine levels shortly after codeine administration and corresponding acute codeine analgesia.

Altering BBB transport was a potential confound in the studies included in this thesis, specifically with regards to the pretreatments used to modify brain CYP2D activity. Studies assessing the impact of nicotine and propranolol on P-gp function are conflicting. Nicotine, and its metabolite cotinine, did not alter P-gp mediated ATPase activity in vitro, suggesting no interaction with P-gp transport; however, nicotine treatment of rats for 14 days by minipump increased saquinavir (a protease inhibitor and P-gp substrate) penetration into the brain (Manda et al., 2010; Wang et al., 2005b). Propranolol dose-dependently decreased digoxin transport in monolayers of Caco-2 (human colon carcinoma) cells, indicative of P-gp inhibition (Bachmakov et al., 2006). However, our findings suggest that nicotine and propranolol, given in the pretreatment paradigms employed in chapters 1 and 2, did not alter BBB transport due to 1) the timing of pretreatment (nicotine and propranolol were administered 8 and 24 hours, respectively, prior to codeine and morphine), and 2) a lack of effect of nicotine and propranolol pretreatment on morphine analgesia.

5.2.1.2 Oxycodone

Compared to codeine and morphine, the contribution of brain CYP2D metabolism to oxycodone-induced analgesia is more complex. Oxycodone is metabolized extensively by CYPs, primarily by CYP3A4 into noroxycodone (45–47% of an oxycodone dose) and CYP2D6 into oxymorphone (11%), which are further metabolized by CYP2D6 and CYP3A4, respectively, into noroxymorphone (14%) (Lalovic et al., 2006; Lalovic et al., 2004). Similar to other opioids, the CYP2D6-mediated metabolism of oxycodone produces a compound with greater affinity for the MOR (Lalovic et al., 2006). Using radioligand displacement studies with membrane-expressed ORs (from Chinese hamster ovary (CHO-K1) cells), oxymorphone displayed a greater
displacement $K_i$ than oxycodone for the MOR (0.36 nmol/L, compared to 16.0), KOR (148 nmol/L, compared to >1000), and DOR (118 nmol/L, compared to >1000) (Klimas et al, 2013; Lalovic et al, 2006). The affinity of oxymorphone for MOR specifically, compared to oxycodone, noroxycodone, and noroxymorphone, respectively, was 44-, 158-, and 16-fold greater, while the EC$_{50}$ of oxymorphone-stimulated GTP$_{\gamma}$S binding to MOR $G_\alpha$ proteins was 8-, 45-, and 4-fold greater (Klimas et al, 2013; Lalovic et al, 2006).

Based on receptor activity alone, our initial hypothesis in chapter 3, as with chapter 1, was that increasing the brain CYP2D-metabolism of oxycodone to oxymorphone would increase oxymorphone levels from oxycodone and increase analgesia. However, preclinical and human studies show little evidence for a role of hepatic CYP2D metabolism of oxycodone to oxymorphone in analgesia. Inhibiting rat CYP2D with i.p. quinine did not alter oxycodone analgesia, while inhibiting human CYP2D6 with oral quinidine or paroxetine reduced plasma oxymorphone levels from oxycodone but did not alter oxycodone analgesia (Cleary et al, 1994; Gronlund et al, 2010). In an analysis of studies assessing oxycodone metabolism in vivo, mean oxycodone levels were repeatedly calculated as the primary contributor to analgesia after oral (83.0% of analgesia due to oxycodone) or intravenous (95.3%) oxycodone administration, as well as with inhibition of the CYP3A4 (71.43%) or CYP2D6 (93.7%) metabolic pathways (Klimas et al, 2013). This may be due, in part, to oxycodone being a relatively potent MOR agonist (5-fold less than morphine) therefore, unlike codeine, oxycodone does not require CYP2D activation to elicit analgesia (Klimas et al, 2013; Lalovic et al, 2006). In chapter 3, in contrast to codeine-induced analgesia in chapter 1, inhibiting brain CYP2D increased brain oxycodone levels and corresponding oxycodone-induced analgesia, while inducing brain CYP2D levels increased brain oxymorphone levels from oxycodone and decreased analgesia.

Oxycodone readily crosses the BBB into the CNS while oxymorphone has low BBB permeability (Bostrom et al, 2006; Klimas et al, 2013; Lalovic et al, 2006; Riley et al, 2008). Using in vivo brain microdialysis and pharmacokinetic-pharmacodynamic modelling in rats, there was a 1.6-fold smaller unbound brain-to-blood ratio of oxymorphone after intravenous oxymorphone (1.90), than of oxycodone after intravenous oxycodone (3.01) (Bostrom et al, 2006; Sadiq et al, 2013). Also, the influx clearance ($CL_{in}$) of oxymorphone was 26.5-fold smaller than oxycodone (72 µl/min*g
compared to 1910 µl/min*g) (Bostrom et al, 2006; Sadiq et al, 2013; Tunblad et al, 2003). While oxymorphone may be actively influxed into the CNS (due to a high influx clearance and unbound brain-to-blood ratio), this appears to occur at a significantly slower rate than for oxycodone. Oxymorphone concentrations produced by peripheral metabolism of oxycodone are very low in humans; healthy volunteers given 20 mg of oxycodone produced peak oxymorphone plasma concentrations of 0.82 ng/ml, compared to 23.2 ng/ml of oxycodone (Kaiko et al, 1996). Extrapolating from this dose in plasma using values for protein binding and the unbound brain-to-blood ratios from literature yields an estimated brain concentration of 1.4 ng/ml oxymorphone, compared to 38 ng/ml oxycodone, which is much lower than an estimated oxymorphone EC₅₀ of 63 ng/ml in brain (Bostrom et al, 2006; Kaiko et al, 1996; Sadiq et al, 2013). While this explains why oxymorphone produced peripherally does not contribute to oxycodone analgesia, it fails to explain why increasing oxymorphone produced centrally (i.e. inducing brain CYP2D) results in a decrease in oxycodone analgesia; therefore, additional considerations are required.

Differences in BBB efflux may contribute to the mechanism by which increased brain oxymorphone production, via inducing brain CYP2D, resulted in decreased oxycodone analgesia. If oxymorphone were effluxed more rapidly than oxycodone, then increasing the amount of oxycodone metabolized to oxymorphone through the CYP2D pathway may expedite the removal of oxycodone from the CNS by efflux. Through pharmacokinetic-pharmacodynamic modelling in rats, oxycodone exhibited an efflux clearance (CL_{out}) 16-fold greater than oxymorphone (630 µl/min*g compared to 39 µl/min*g) (Bostrom et al, 2006; Sadiq et al, 2013). However, this is likely due to much higher brain levels of oxycodone than oxymorphone (influxed and/or produced centrally), as oxycodone exhibits a net influx clearance 17-fold greater than oxymorphone (560 µl/min*g compared to 33 µl/min*g) (Bostrom et al, 2006; Sadiq et al, 2013). In rats infused with the P-gp inhibitor PSC833, there was no difference in brain oxycodone levels, plasma oxycodone pharmacokinetics, or oxycodone analgesia, compared to vehicle controls, indicating oxycodone is not a P-gp substrate (Bostrom et al, 2005). Similarly, oxymorphone did not alter in vitro P-gp mediated ATPase activity, indicating that it may be neither a P-gp substrate nor an inhibitor (Metcalf et al, 2014). Therefore, while there do not appear to be major differences in the CNS influx or efflux between oxycodone and oxymorphone, the net differences in brain/plasma partitioning and clearance may be sufficient to negate the effect of changes in brain oxymorphone
levels on oxycodone analgesia under these conditions. Any impact of P-gp on oxymorphone efflux in vivo could be tested by inducing brain CYP2D concurrently with inhibition of P-gp (e.g. with infusion of the P-gp inhibitor PSC833), and assessing the impact on oxycodone analgesia. Although in vitro studies suggest oxymorphone is not a substrate of P-gp, if inducing brain CYP2D metabolism of oxycodone to oxymorphone in vivo decreases oxycodone analgesia due to more rapid efflux of oxymorphone, this should be blocked by P-gp inhibition.

There are numerous other factors that may account for changes in central oxymorphone levels not contributing to oxycodone analgesia. While oxycodone acts primarily on MORs, oxymorphone shows high affinity for MORs, KORs, and DORs (Lalovic et al, 2006). Agonists of DOR and KOR can increase MOR-agonist response in vivo; intracerebroventricular injection of the DOR-agonist DPDPE increased morphine-induced EEG bursts in rats, while repeated subcutaneous administration of the KOR-agonist (-)U-50,488H increased MOR activity in mice (Narita et al, 2003; Stamidis and Young, 1992). Therefore, oxymorphone activation of KORs and DORs could increase oxycodone activation of MORs, contributing to oxycodone’s predominant role in analgesia. However, after brain CYP2D induction, increased brain oxymorphone levels from oxycodone did not increase oxycodone-induced analgesia, suggesting that under these conditions, KOR and DOR activation by oxymorphone is unlikely to play a role. Differences in short-term MOR regulatory events, like desensitization of receptors, may also contribute to agonist-specific differences in response (Whistler et al, 1999). In rat locus coeruleus neurons in vitro, oxymorphone evoked a large change in potassium channel current (348 pA) but rapid desensitization (to 69% of the peak), whereas oxycodone evoked a current (278 pA) similar to morphine but without desensitization (<1% of peak) (Virk and Williams, 2008). In chapter 3, increased brain oxymorphone levels from induced brain CYP2D metabolism of oxycodone may have resulted in rapid MOR desensitization, and thereby decreased the ability of oxycodone to activate, and thereby produce analgesia at, MORs.

We do not know the precise reason that inhibiting brain CYP2D increased oxycodone levels, without altering oxymorphone levels, while inducing brain CYP2D increased oxymorphone levels, without altering oxycodone levels. Our behavioural findings suggest that oxycodone (brain levels increased with increased analgesia), but not oxymorphone (brain levels increased with decreased
analgesia), was predominantly responsible for oxycodone-induced analgesia. However, after inducing brain CYP2D, we observed no corresponding decrease in brain oxycodone levels with decreased analgesia. Difficulties in compound detection may explain some of this discrepancy. Oxymorphone levels were below the limit of quantitation (0.1 ng/ml) in samples collected from 0 – 60 min after oxycodone administration in 78% of vehicle pretreated animals (7/9), 86% of propranolol pretreated animals (6/7), and 71% of nicotine and propranolol pretreated animals (5/7). In comparison, oxymorphone was detected in all animals pretreated with nicotine (5/5), further strengthening the conclusion that nicotine pretreatment induced the brain CYP2D metabolism of oxycodone to oxymorphone. However, this fails to explain why there was no observed decrease in brain oxycodone levels after inducing brain CYP2D. One potential explanation is that the oxycodone (or oxymorphone) levels collected in nucleus accumbens did not reflect those in regions mediating analgesia. The relative levels of oxycodone and oxymorphone and other metabolites sampled from the nucleus accumbens did not differ from those sampled from the cerebral ventricles, suggesting that oxycodone and its metabolite levels were homogenous across brain regions. However, rat brain stem, which contains regions involved in opioid-modulation of pain (or antinociception) transmission (e.g. PAG, RVM, dorsal root ganglia), expresses roughly 2.1-fold more CYP2D protein than rat striatum, which includes the nucleus accumbens (Ossipov et al, 2010; Yue et al, 2008). More CYP2D protein in the brain stem may have produced an isolated microenvironment of higher brain CYP2D activity, and therefore oxycodone metabolism, accompanied by changes in oxycodone levels that were not detected in the nucleus accumbens or cerebral ventricles.

5.2.2 The potential impact of brain CYP2D on other opioids
The opposing impacts of brain CYP2D on codeine (by formation of active metabolite) and oxycodone (by removal of active parent) analgesia in the two acute administration models in this thesis provide a basis for predicting the role of brain CYP2D in analgesia from other opioids. For example, the opioid tramadol is metabolized primarily by CYP2D6 to O-desmethyltramadol (O-DSMT, 50% of urinary recovery), which has a 300-fold greater affinity for the MOR than the parent tramadol and is therefore believed to be responsible for tramadol’s analgesic effects (Grond and Sablotzki, 2004; Wang et al, 2015). Consistent with this hypothesis, Malaysian pain-patients genotyped as CYP2D6 UM and EMs had greater tramadol clearance (to O-DSMT) and MOR-
related adverse-effect profiles than IMs (Gan et al., 2007). Furthermore, at 10 min after oral tramadol administration in rats (time of approximate peak brain tramadol levels), the ratio of tramadol/O-DSMT was 10 to 30-fold greater in brain than plasma, suggesting that, compared to tramadol, O-DSMT has difficulty crossing the BBB (Tao et al., 2002). Tramadol, but not O-DSMT, levels in rat correlated between cerebrospinal fluid and plasma after intraperitoneal tramadol administration (Wang et al., 2015). The difference in MOR affinity between parent and metabolite, the role of CYP2D metabolism, and the apparent differences in BBB permeability, suggest that central CYP2D-mediated metabolism of tramadol to O-DSMT likely functions as an activation pathway eliciting analgesia. This was assessed in vivo in rats; inhibiting brain CYP2D increased tramadol t1/2 in brain and decreased tramadol analgesia and brain O-DSMT levels after intraperitoneal tramadol administration, while inducing brain CYP2D decreased tramadol t1/2 in brain and increased tramadol analgesia and brain O-DSMT levels (Wang et al., 2015). Thus, it appears that the role of brain CYP2D in analgesia is similar for tramadol and codeine, functioning as an activation pathway.

Hydrocodone, a semi-synthetic opioid compound with structural similarities to oxycodone, is metabolized primarily through 6-keto-reduction to dihydrocodeine, but also through CYP2D6 to hydromorphone (0-5% of urinary recovery), a compound with 33-fold greater MOR affinity than hydrocodone (Chen et al., 1991a; Cone et al., 2013; Smith, 2011). Nevertheless, the influence of CYP2D6 metabolism (e.g. genetic variation in CYPD6 activity) on hydrocodone response is unclear. In two independent clinical cases, patients genotyped as CYP2D6 PMs were able to achieve analgesia from hydrocodone, but not codeine, suggesting that the CYP2D6 pathway influences analgesia from hydrocodone and codeine differently (Foster et al., 2007; Susce et al., 2006). This may be due to hydrocodone itself possessing activity at MORs, with an in vitro MOR displacement Ki 16-fold less than morphine and 2.4-fold less than oxycodone, but 12.5-fold greater than codeine (Chen et al., 1991a). However, in a prospective observational study, hydrocodone effectiveness was decreased in individuals concurrently using CYP2D6 substrates and inhibitors (Monte et al., 2014). While hydrocodone has been identified as a P-gp substrate in vivo, hydromorphone transport across the BBB is unknown (Somogyi et al., 2007). Since hydrocodone activates MORs, undergoes minor CYP2D-mediated metabolism to the more potent hydromorphone, and its analgesia is not greatly influenced by peripheral CYP2D metabolism, the
relative role of parent and metabolite in hydrocodone analgesia apparently resembles that of oxycodone more closely than for codeine and tramadol. This suggests that brain CYP2D-mediated metabolism of hydrocodone to hydromorphone may play an inactivation role in mediating analgesia, similar to that observed for oxycodone.

Fentanyl, a synthetic opioid, is predominantly converted by CYP3A4-mediated N-dealkylation to the nontoxic and inactive metabolite norfentanyl (76% of urinary excretion by intravenous administration), and is not metabolized by CYP2D6 (Smith, 2011). Inhibiting CYP3A4 in human liver microsomes in vitro with ketoconazole and erythromycin reduced fentanyl metabolism (by 90%), while inhibiting CYP2D6 with quinidine had no effect (Feierman and Lasker, 1996). Therefore, neither CYP2D6 genetic polymorphism nor variable brain CYP2D6 metabolism would be expected to play a role in altering fentanyl metabolism and analgesia. However, like morphine and oxymorphone, which are not further metabolized by CYP2D6, fentanyl analgesia in vivo could serve as a useful control for off-target effects of brain CYP2D inhibitor and/or inducer pretreatments.

5.3 Brain CYP2D and chronic opioid administration

5.3.1 Absolute vs. proportional decrease in codeine analgesia
One of the novel findings from chapter 2 was that the rate of development of codeine and morphine tolerance, or decrease in peak analgesic response across repeated administration, correlated with acute peak analgesic response. We hypothesized that an increase in acute codeine analgesia via induction of brain CYP2D would result in one of the following outcomes: A) either a similar absolute decrease in analgesia across time with a reduced proportional decrease in analgesia and therefore a longer time to develop tolerance (Figure 35, Option A compared to reference group), or B) a similar proportional decrease in analgesia across time, and therefore an increased absolute decrease in analgesia with similar time to develop tolerance (Figure 35, Option B). In support of option B, we demonstrated in chapter 2 (Figure 18c) that inducing brain CYP2D with nicotine pretreatment increased acute codeine response (43.1 vs. 26.9% MPE, p<0.01), producing a similar proportional (28 vs. 33%), but greater absolute (10.0 vs. 3.8% MPE, p<0.03), decrease in analgesia over the first four days of codeine administration (Figure 35, Option B). We
are unaware of any study in the literature that has described analgesic tolerance in this manner, therefore the consequences of this finding are unclear. Our results suggest that a greater acute analgesic response predicts a greater decrease in response (and therefore perception of analgesia) over time per dose of codeine. Consistently, when acute analgesia was increased by giving larger doses of codeine, there was a similar relationship observed between acute analgesia and the rate of tolerance (Figure 23a). The impact of this finding on repeated/chronic opioid use, such as codeine or oxycodone, may be important in predicting adverse outcomes from developing tolerance.

It is important to consider the impact of an increased rate of opioid tolerance on opioid-taking behaviours. Based on the models in Figure 35 and our findings in chapter 2, we propose that individuals who experience greater acute analgesia from an opioid (in our model, codeine or morphine) would reach half-maximal response at the same time of opioid-taking as those with lower acute analgesic effect (Figure 35, purple and grey dotted lines). Individuals who experience greater acute analgesia, by having greater brain CYP2D activity or beginning opioid treatment at (or illicitly taking) a higher opioid dose, would experience a greater decrease in response than those receiving a lower dose, and would be potentially more likely to escalate dose and to a greater extent. Tolerance to opioid-induced side effects like respiratory depression can cause an increase in mortality, for example when individuals take the same dose of opioid in a different environment, or when individuals relapse after an extended period of abstinence during which tolerance is lost (Cahill et al, 2016). For example, rats injected with a high dose of heroin in an environment associated with prior heroin injections (associated context cues) experienced less mortality (32.4%) than rats injected in an environment not associated with prior heroin injections (64.3%, different context cues) and rats naïve to heroin (96.4%) (Siegel et al, 1982).
Figure 35: Initial hypotheses (option A and B, compared to reference in grey) of the influence of increased acute codeine analgesia on the rate of development of tolerance. Compared to the reference group (grey) showing decreased analgesia across repeated codeine administration, we hypothesized two outcomes (option A and B) for the impact of increasing acute codeine analgesia (by inducing brain CYP2D or increasing codeine dose, shown by red arrow) on the rate of codeine tolerance. In option A, increasing acute analgesia may produce a smaller proportional, but similar absolute, decrease in analgesia compared to the reference group, resulting in a greater time to tolerant or days to 50% response (blue dashed line) with daily codeine administration. Alternatively, in option B, increasing acute analgesia may produce a similar proportional, but greater absolute, decrease in analgesia compared to the reference group, resulting in the same time to tolerant or days to 50% response (purple dashed line) with daily codeine administration.

In a longitudinal prospective study of patients receiving opioids for cancer pain, individuals treated with lower (15-20 mg, n=14) compared to higher (30-60 mg, n=16) doses of morphine, increased morphine dose across three weeks to 57.1 mg and 83.4 mg, respectively (2.9-fold and 1.9-fold increases, Figure 36) (Corli et al, 2015). Similarly, individuals treated with lower doses of oxycodone (15-30 mg, n=19), compared to higher (40-80 mg, n=25), increased oxycodone dose across three weeks to 48.9 mg and 87.2 mg, respectively (1.8-fold for both) (Corli et al, 2015). This suggests that initiating opioid treatment at both low and high doses can produce dose-escalation. However, acute analgesic response to the initial doses used was difficult to ascertain from the study; it’s unknown if individuals who received high acute doses of opioid were experiencing greater analgesia (Corli et al, 2015). Assessing analgesic response after first dose
through visual analogue scales or pain indices could be used to assess the relationship between acute effect and dose escalation that we propose from our study.

**Figure 36**: Dose-escalation of morphine and oxycodone after 21 days of opioid treatment in individuals receiving treatment for chronic cancer pain. Patients in a longitudinal prospective study receiving opioid treatment for cancer pain were split evenly into low and high initial-dose groups. Starting doses on day 1 and final doses on day 21 were recorded and averaged. Individuals initiating treatment on low-dose morphine escalated to 2.9-fold the dose ion day 21, compared to 1.9-fold in those initiating on high-dose morphine. Individuals initiating treatment on low-dose oxycodone escalated to 1.8-fold the dose on day 21, compared to 1.8-fold in those initiating on high-dose oxycodone. Adapted from Corli et al. 2015.

Beyond opioid-taking behaviour, the findings in chapter 2 raise the question of the significance of a relative vs. absolute decrease in analgesia over time to the neurobiology of tolerance. First, there are agonist-specific short- and long-term neural adaptations involved in opioid tolerance (introduced in section 1.1.4.2) that could be investigated with respect to greater acute MOR agonist exposure producing a greater relative neural response. Following receptor binding, MOR phosphorylation by G-protein receptor kinases (GRKs) initiates desensitization and internalization of the MOR, which involves the association of β-arrestin proteins uncoupling MOR from G-proteins (Dumas and Pollack, 2008; Koch and Hollt, 2008). These processes correlate with agonist-induced desensitization and decreased functional receptor response. Morphine does not initiate GRK-mediated MOR phosphorylation or MOR endocytosis (Keith et al, 1996; Sternini et
al, 1996; Zhang et al, 1998). Instead, morphine has been suggested to produce analgesic tolerance as a result of compensatory mechanisms counteracting receptor function (Koch and Hollt, 2008). While acute morphine exposure inhibits adenylyl cyclase activity, chronic exposure produces a counter-regulatory increase in adenylyl cyclase and resulting cAMP levels (adenylyl cyclase superactivation) (Nevo et al, 1998; Sharma et al, 1975a; Sharma et al, 1975b). In support of this, subcutaneous morphine given BID to rats at a dose that produced analgesic tolerance (decreased tail-flick response) and physical dependence (increased withdrawal score) induced coupling of stimulatory G-protein (G_s) to MOR in rat striatum and spinal cord (Wang et al, 2005a). Alterations in G-protein coupling were not due to changes in expression of MOR or G-proteins, and decreasing analgesic tolerance and physical dependence by co-pretreatment with an ultra-low dose of naloxone (non-selective OR antagonist) decreased G_s-coupling (Wang et al, 2005a). Furthermore, chronic morphine induced glial-cell (i.e. astrocytes and microglia) activation, inflammation, and analgesic tolerance were increased in animals given daily intrathecal morphine, and decreased by co-pretreatment of an ultra-low dose of naltrexone (MOR antagonist) (Mattioli et al, 2010). Assessing differences in G_s-MOR coupling (by immunoprecipitation) or gliosis (by immunohistochemistry) in rats tolerant to codeine, after different acute analgesic responses (either by induction of brain CYP2D-activation to morphine or by increased doses), may be useful in associating quantifiable neural alterations with the correlation of acute analgesic response to rate of tolerance seen in chapter 2.

Oxycodone produces potent analgesic tolerance; in rats with induced inflammatory pain given intraperitoneal oxycodone BID for 8 days, there was a 10-fold rightward shift in the dose response curve of oxycodone-induced mechanical hyperalgesia on day 9 compared to day 1 (Thorn et al, 2017). Since we demonstrated in chapter 2 that acute response to codeine impacted the rate of codeine tolerance, and in chapter 3 that brain CYP2D-mediated metabolism of oxycodone impacts acute analgesic response, the development of oxycodone tolerance may similarly develop as a function of acute oxycodone analgesia. Decreasing acute oxycodone analgesia by inducing brain-CYP2D metabolism of oxycodone to oxymorphone may result in a decreased rate of oxycodone tolerance. Compared to morphine, which promotes MOR desensitization without internalization, the mechanism of oxycodone tolerance and extent of oxycodone-induced desensitization and internalization of MORs is unclear (Allouche et al, 2014). Oxycodone does not strongly promote
MOR phosphorylation by c-Jun N-Terminal Kinase, or recruit GRK-arrestin pathways (GRK-induced β-arrestin coupling), nor does it evoke MOR internalization (demonstrated with rat MORs in human embryonic kidney [HEK293] cell lines) (Koch et al., 2009; McPherson et al., 2010; Williams et al., 2013). Therefore, similar to codeine and morphine-induced tolerance, oxycodone tolerance may occur due to adaptive processes like adenylyl cyclase superactivation.

5.3.2 Implications of brain CYP2D in opioid abuse liability
As discussed in section 1.3.3, the abuse potential of opioid compounds depends on numerous factors, including the speed of onset of drug response, duration of response, and net positive and negative drug effects (Wightman et al., 2012). Like analgesia, the subjective effects of opioid use (e.g. euphoria and elation) are mediated by action at the MORs, and therefore sources of variability in analgesic response may also impact abuse liability. Specifically, analogous to the analgesic effects, the initial reinforcing effects of codeine and oxycodone may be mediated by their CYP2D metabolism within the brain prior to peripheral metabolites entering the CNS (discussed in sections 5.2.1.1 [codeine] and 5.2.1.2. [oxycodone]).

Pharmacokinetic assessments of opioid abuse potential often use a measured abuse quotient, defined as the ratio of maximum plasma drug concentration ($C_{MAX}$) over the time to maximum concentration ($T_{MAX}$) (Harris et al., 2014). A high abuse quotient indicates increased likelihood of drug liking and has been used as a standard comparator for abuse-deterrent opioid formulations (Perrino et al., 2013; Webster et al., 2016). While our studies did not determine the full kinetic profile of codeine and oxycodone, we can adapt this measure using the peak analgesic effect ($E_{MAX}$) and time to peak analgesia in animals across brain CYP2D inhibitor and/or inducer pretreatment groups. In chapter 1, the predicted codeine abuse quotient was greatest in rats pretreated with the brain CYP2D specific inducer nicotine (5.02% MPE/min), and lowest in rats pretreated with the brain CYP2D specific inhibitor propranolol (1.4% MPE/min; Figure 37). This relationship was reversed in chapter 3, where the predicted oxycodone abuse quotient was greatest in rats with inhibited brain CYP2D (3.4 %MPE/min), compared to induced brain CYP2D (1.5 %MPE/min; Figure 37). This suggests that, similar to the effect of brain CYP2D on analgesia, individuals with greater brain CYP2D activity may be at greater risk for codeine abuse, due to the
rapid activation of codeine to morphine, but at a lesser risk for oxycodone abuse, due to the rapid metabolism to, and subsequent efflux of, oxymorphone.

![Figure 37: Modified abuse quotient (E_{MAX}/T_{MAX}) for codeine and oxycodone after brain CYP2D inhibitor and/or inducer pretreatment.](image)

Rats pretreated (PT) with the brain CYP2D specific inducer nicotine had a greater codeine abuse quotient, while rats pretreated with the brain CYP2D specific inhibitor propranolol had a greater oxycodone abuse quotient. Abuse quotient was calculated using E_{MAX} and T_{MAX} values obtained in McMillan and Tyndale 2015, and McMillan et al. 2017.

The impact of brain CYP2D on codeine and oxycodone abuse can be determined through animal models of abuse liability; rats does-dependently self-administer codeine and oxycodone (Mavrikaki et al, 2017; O'Connor et al, 2011). However, elucidating this in human populations is more difficult, owing to the lack of literature assessing a direct role of central metabolism on drug response, as well as the difficulty in isolating the effect of xenobiotic inducers on brain, compared to liver, CYP2D6. For example, as discussed in section 1.4.4.2, CYP2D6 levels are higher in the post-mortem brains of smokers compared to non-smokers, while liver CYP2D6 levels do not differ (Mann et al, 2008; Miksys et al, 2002; Miksys and Tyndale, 2004; Zanger and Schwab, 2013). However, cigarette smoking is strongly associated with greater drug taking overall, including opioids; in a sample of over 600 tertiary pain clinic patients, 82% of smokers used opioid medications compared to 50% of non-smokers (Eckhardt et al, 1994; Mackey et al, 2016). The increased prevalence of devices that deliver nicotine alone, like electronic cigarettes, particularly
in adolescents, suggests a unique population of individuals who may experience nicotine-induction of brain CYP2D6 and thereby altered CNS-acting CYP2D6 substrate drug response, like opioids (Dutra and Glantz, 2014). Comparing e-cigarette users to non-users, genotyped for CYP2D6 activity, on their response to CNS-acting CYP2D6 substrates used clinically could be useful in elucidating an effect of nicotine on brain CYP2D6 metabolism.

5.4 Functional impact of brain CYP2D6 metabolism of opioids in humans

5.4.1 Brain CYP2D6 and the opioid epidemic
The findings in this thesis can be considered broadly in the context of the current opioid epidemic, primarily with regard to the potential for brain CYP2D6 metabolism of opioids to influence opioid response in humans, and what outcomes would be most impacted by variation. As discussed in section 1.2.2.2, human CYP2D6 is highly genetically polymorphic, resulting in stratification of individuals into metabolism phenotypes with measurable differences in CYP2D6 drug metabolism and resultant response (Zanger et al, 2004). The experimental paradigms used throughout this thesis were developed to inhibit and induce brain CYP2D selectively, without impacting hepatic CYP2D. We showed that there was no impact of brain CYP2D inhibitor and/or inducer pretreatments on plasma morphine levels after i.p. codeine (chapter 1); on ex vivo hepatic CYP2D activity or the plasma codeine CYP2D metabolic ratio (morphine to codeine) after p.o. codeine (chapter 2); or on ex vivo hepatic CYP2D activity or the plasma oxycodone CYP2D metabolic ratio (oxymorphone to oxycodone) after p.o. oxycodone (chapter 3). Due to genetic polymorphism, however, humans possess a wide-range of CYP2D6 activity in the liver that may translate to the brain. CYP2D6 PMs have observable differences in brain function and behaviour, which may be due, in part, to altered CNS metabolism of endogenous signaling molecules (Kirchheiner et al, 2011). Furthermore, in CYP2D6 PMs homozygous for the gene deletion CYP2D6*4, there was no detectable CYP2D6 in liver or enzymatic activity in vivo, and no CYP2D6 detected in brain (Miksys et al, 2002). This suggests that genetic variation in CYP2D6 (e.g. decreased function, duplication, etc.) may similarly impact brain CYP2D6 enzyme activity. To elucidate the impact of brain CYP2D6 metabolism on opioid compounds clinically, the combination of genetics, xenobiotic inducers, and other influences on brain metabolism must be considered.
Brain CYP2D6 metabolism in humans may explain some of the existing variability in opioid levels and response within or between CYP2D6 genotypes (or phenotype groups). In vivo phenotyping was used initially to categorize individuals into four distinct phenotype groups; due to the large number of allele combinations per phenotype group, activity scores for each allele have been employed to better represent CYP2D6 activity (Gaedigk and Coetsee, 2008; Gaedigk et al, 2008). However, variation still exists in both peripheral metabolism kinetics and subjective drug response within these CYP2D6 activity score groups, particularly in CYP2D6 IMs and EMs. For example, in a cohort of women prescribed codeine post-operatively, variability in total pain scores at day 2 after codeine was highest in those classified as CYP2D6 IMs (coefficient of variation = 0.44) or EMs (0.46) compared to PMs (0.32) and UM (0.19), despite the EMs and IMs groups containing the greatest number of individuals (VanderVaart et al, 2011). Some of this variation may be explained by differences in peripheral codeine and morphine levels due to a range of both codeine dosing by weight (range of 0.4-11.1 mg/kg in IMs and 0.8-10.8 mg/kg in EMs) and CYP2D6 diplotypes in each predicted phenotype (12 in IMs and 3 in EMs) (VanderVaart et al, 2011). However, variation in the brain CYP2D6 metabolism of codeine (for example through exposure to xenobiotic brain CYP2D6 inducers or inhibitors) may also account for some of this within-phenotype variability in codeine response.

Our findings in chapters 1 and 3 strongly suggest an impact of brain CYP2D6 on acute opioid response. Opioids are routinely prescribed in an acute setting, with emergency department visits being a major source of prescriptions; from 2001 to 2010 in the U.S. the percentage of emergency department visits that resulted in opioid prescriptions increased from 20.8% to 31.0% (Mazer-Amirshahi et al, 2014; Volkow et al, 2011). One prospective study in 2015 in the U.S. found that 90.5% of opioid-naïve patients (581 of 642) were prescribed an opioid after common outpatient general surgery procedures (Hill et al, 2017). Our data suggests that individuals exposed to brain CYP2D6 inducers (e.g. nicotine or alcohol) who were then administered opioids acutely would experience increased analgesia from codeine, and decreased analgesia from oxycodone. This may also manifest as increased or decreased subjective effects of each opioid, potentially impacting likelihood of abuse, as well as adverse events.
5.4.2 Potential sex differences in the brain CYP2D6 metabolism of opioids

Investigating CNS-acting drug response and CYP2D metabolism represents an interesting intersection with known sex differences between males and females. Population-based studies show that women are more likely to experience, and more frequently report, chronic pain symptoms than men (Lee and Ho, 2013). Based on the Canadian Alcohol and Drug Use Monitoring Survey, opioid use is higher among Canadian women (18.3%) than men (15.5%), while prescription opioid abuse in U.S. treatment centres was reported to be higher among women (15.4%) than men (11.1%) (Canada, 2015; Green et al, 2009). Similarly, female rats show a greater vulnerability to drug abuse than males in numerous paradigms (Anker and Carroll, 2011). Analgesia, drug reward, and vulnerability to relapse additionally vary with the estrous cycle in female rats, although findings are inconsistent. Ovariectomized female rats given exogenous estradiol had increased acquisition of heroin self-administration and heroin-maintained responding; however, there was no effect of estrous cycle in sham-operated rats (Roth et al, 2002). In contrast, mean heroin infusions were lower in female rats in proestrus (high estradiol and progesterone) than during estrus and meta/diestrus (Lacy et al, 2016). Little is known about differences in acute response to codeine and oxycodone between males and females. In comparing the antinociceptive potency (ED\textsubscript{50}) of multiple opioids in the warm water tail withdrawal assay, male rats displayed greater antinociceptive potency (lower ED\textsubscript{50}) of hydrocodone (1.7-fold), hydromorphone (1.6-fold), and morphine (2.8-fold) than female rats, but there was no difference in ED\textsubscript{50} of oxycodone and codeine (Peckham and Traynor, 2006). Furthermore, male rats self-administer more oxycodone than females at FR1 schedules, but females self-administer more oxycodone than males at higher doses, although the specific stage of estrous cycle was not examined (Mavrikaki et al, 2017)

The influence of sex and gender on CYP2D6 activity is also conflicting, as CYP2D6 activity has been reported as faster and slower in females compared to males (Soldin et al, 2011; Soldin and Mattison, 2009). Among females, this may vary based on the estrous cycle; estrogen decreases CYP2D6 activity in vitro, and may decrease CYP2D6 activity in humanized Tg-\textit{CYP2D6} mice in vivo by enhancing SHP repression of HNF-4α activation of \textit{CYP2D6} (section 1.2.2.2.2) (Pan and Jeong, 2015; Pepper et al, 1991). In a large cohort of \textit{CYP2D6} EMs, females had slightly lower (1.2-fold) urinary dextromethorphan/dextrorphan ratios than males, indicative of increased
CYP2D6 activity, while there was no effect of co-use of oral contraceptives (Hagg et al, 2001). Furthermore, little is known regarding sex differences in brain CYP2D. Pain and opioid response are sexually dimorphic; as hepatic CYP2D6 activity does not vary greatly between sexes, this suggests an influence of sex and/or sex hormones elsewhere on opioid response. Sex differences in brain CYP2D6 activity may account for some of this variability.

Our lab has begun to investigate sex differences in brain CYP2D activity in rats, along with the corresponding impact on brain opioid metabolism and response, in vivo. Pilot data has shown that females have lower ex vivo brain CYP2D activity than males (0.6-fold), with no difference in ex vivo liver CYP2D activity (Figure 38). In support of this, female rats had greater brain oxycodone levels and lower brain oxycodone CYP2D-metabolic ratios (oxymorphone/oxycodone) than males assessed by in vivo brain microdialysis after oral oxycodone administration (Figure 38). While these findings are preliminary, they suggest that decreased brain CYP2D activity in females may reduce brain oxycodone metabolism in vivo, resulting in increased oxycodone analgesia. This is in line with the finding that female rats self-administer more oxycodone than males, suggesting central metabolism of oxycodone to oxymorphone is decreased, yielding greater subjective effects of oxycodone; this is, however, inconsistent with the finding that males and female rats do not exhibit different codeine and oxycodone antinociceptive ED$_{50}$ (Mavrikaki et al, 2017; Peckham and Traynor, 2006). Additional studies are in progress to elucidate the full impact of sex on oxycodone analgesia, peripheral oxycodone levels and metabolism, as well as phase-specific effects of the female estrous cycle.
5.4.3 Endogenous opioid pain thresholds

Endogenous morphine-like opioids, including endorphins and enkephalins, are released under conditions such as surgical, pathogenic, psychological, and physical stress and may be involved in the modulation of nociception and pain sensitivity through activation of ORs (Candiotti et al., 2009; Guarna et al., 2002). For example, in a group of patients undergoing cardiac bypass surgery who received no morphine for post-surgery pain, there was a 13.8-fold increase in plasma morphine concentrations 24 hr post-surgery (80 pg/ml to 1106 pg/ml) (Brix-Christensen et al., 1997). CYP2D6 may contribute to the biosynthesis of endogenous morphine (endorphins) in vivo. Incubating human polymorphonuclear white blood cells with the endogenous morphine precursor tyramine dose-dependently increased morphine concentrations in vitro (Zhu et al., 2005). Morphine synthesis was diminished when white blood cells were coincubated with tyramine and the CYP2D6 substrate bufuralol, and was blocked entirely when coincubated with tyramine and quinidine (CYP2D6 inhibitor) (Zhu et al., 2005). The presence of CYP2D6 enzyme was supported by RT-PCR, suggesting CYP2D6 was expressed in human white blood cells and capable of synthesizing endogenous morphine from exogenous precursors (Zhu et al., 2005).

Interindividual differences in CYP2D6 metabolism (e.g. genetic polymorphism) may impact the production of endogenous opioids, and therefore functional outcomes like pain thresholds.
*CYP2D6* PMs had higher sensitivity to pressure pain than EMs indicating less tolerance to tonic pain; a higher fraction of PMs (32%), than EMs (18%), prematurely withdrew their hand during the cold pressure test (Sindrup et al, 1993). In a cohort of females in the U.S. investigated for post-surgical pain, *CYP2D6* PMs experienced severe post-operative pain more frequently than all other phenotype groups suggesting lower pain thresholds (Yang et al, 2012). Additionally, in a separate group of females from the same cohort taking morphine (not a CYP2D6 substrate), *CYP2D6* UMs required less morphine than all other phenotype groups (Candiotti et al, 2009). While there is relatively little literature on this phenomenon, differences in pain sensitivity among *CYP2D6* genotype groups suggests *CYP2D6* may impact endogenous nociception, possibly through endogenous opioid formation.

As discussed briefly in section 1.4.4.3.2, *CYP2D6* expressed in rat brain membranes can metabolize precursors tyramine and 5-methyltryptamine to dopamine and serotonin, respectively, in vitro, while in vivo brain microdialysis studies indicate that this formation pathway may be functional in vivo (Bromek et al, 2010; Bromek et al, 2011; Haduch et al, 2015; Haduch et al, 2013). However, this was investigated after ablation of the classical dopamine and serotonin formation pathways in vivo and with amplification of the role of brain *CYP2D* through exogenous precursor administration; therefore, the extent of brain *CYP2D*’s impact on basal endogenous metabolism is unknown (Bromek et al, 2011; Haduch et al, 2015). The suggested impact of brain *CYP2D6* on endogenous neurochemical regulation, including that found in literature on *CYP2D6* genotype and pain thresholds, infers that brain *CYP2D* may contribute to endogenous opioid homeostasis; individuals exposed to xenobiotic inducers of brain *CYP2D* (e.g. smokers and alcoholics) may have higher pain thresholds due to increased production of endogenous opioids in the CNS. However, in chapter 1 and 3 we saw no effect of our brain *CYP2D* specific inhibitor and inducer pretreatments on baseline nociception (i.e. threshold or sensitivity to a noxious stimulus) and morphine-induced analgesia. Furthermore, in two separate studies assessing electrical pain stimulation thresholds and cold pressor pain tolerance, there was no observable difference in pain sensitivity between smokers and non-smokers (Unrod et al, 2004; Waller et al, 1983). This may be due to peripheral ORs (e.g. in the skin) and peripherally formed endogenous opioids (e.g. from white blood cells) contributing more to pain sensitivity and thresholds than CNS ORs and centrally formed endogenous opioids (Kapitzke et al, 2005).
5.5 Extensions of research

5.5.1 Drug-drug interactions with brain CYP2D

Having shown that brain CYP2D activity impacts codeine analgesia (by formation of the more active metabolite morphine) and oxycodone analgesia (by metabolism of the parent oxycodone to the metabolite oxymorphone), it is likely that drug-drug interactions with substrates or inhibitors of brain CYP2D may impact codeine and oxycodone response in a clinically significant manner (introduced in section 1.2.2.2.2) (Feng et al, 2017). Numerous randomized clinical trials have shown that CNS-acting drugs that are CYP2D6 inhibitors alter peripheral metabolism and response to opioids that are more dependent on metabolic activation by CYP2D6 for analgesic effect (e.g. codeine and tramadol). Administration of the antidepressant paroxetine, a CYP2D6 inhibitor, to CYP2D6 EMs decreased the total plasma concentrations of tramadol’s CYP2D-metabolite O-demethyltramadol, and decreased the hypoalgesic effect of tramadol in experimental pain models (Laugesen et al, 2005). Similarly, in patients hospitalized for acute back pain, administration of the neuroleptic levomepromazine, a CYP2D6 inhibitor, in CYP2D6 EMs decreased the CYP2D6-mediated O-demethylation ratio of morphine to codeine, indicating the likelihood of insufficient codeine pain relief (Vevelstad et al, 2009). Therefore, while there are documented changes in plasma O-demethyltramadol (from tramadol) and morphine (from codeine) levels in these studies, indicative of decreased peripheral CYP2D6 activity, the influence of paroxetine and levomepromazine on brain CYP2D6 activity, among other CNS-acting CYP2D6 inhibitors, may also contribute to the changes in opioid pharmacodynamic effects and response.

Comparatively, drug-drug interactions of brain CYP2D-inhibitors and opioids may produce a greater pharmacodynamic impact on opioids where the contribution of hepatic CYP2D6 to analgesia is negligible or unknown (e.g. oxycodone and hydrocodone). In a randomized clinical trial of healthy individuals, paroxetine reduced total oxymorphone plasma levels from oxycodone (44% reduction in AUC), but did not alter plasma oxycodone levels or response (Gronlund et al, 2010). The individuals in this study were primarily (10 of 12 participants) CYP2D6 EMs; it is possible that interference in brain CYP2D6 metabolism by drug-drug interactions were masked due to the range of CYP2D6 genotypes and therefore CYP2D6 basal activity in CYP2D6 EMs. A randomized control trial, stratifying individuals based on CYP2D6 genotype and oxycodone
response prior to, and following, paroxetine treatment (or another CNS-acting CYP2D6-inhibitor), may be useful for assessing genotype-specific influences of drug-drug interactions on the response to more metabolically-complex opioids like oxycodone.

5.5.2 Imaging brain CYP2D6 in humans
The ability to determine the location, abundance, and activity of CYP2D6 enzyme in the brain in vivo using non-invasive molecular imaging could provide valuable information about brain CYP2D6. In general, positron emission tomography (PET) has been a viable avenue to pursue this, as radiotracers can be designed for catabolism by CYP enzymes (Holland et al., 2013). For example, in separate studies assessing radiotracer labelling specificity in patients with epilepsy, there was an increase in the CYP3A4-mediated metabolism of radiotracers \([^{11}C]\)-deprenyl and \([^{11}C]\)-verapamil, attributed to the potential induction of liver CYP3A4 by anticonvulsant medications (Abrahim et al., 2008; Cumming et al., 1999). However, PET imaging of CYP enzymes has been difficult due to trapping or accumulation of enzymatic products, as well as specificity of radiotracer ligand (Holland et al., 2013). High affinity inhibitors are currently being developed to serve as tracers, particularly for measuring enzymes mediating intracellular signal transduction in the brain (Holland et al., 2013). For example, CYP19A1 is expressed in human brain, and has been shown to catalyze the formation of estradiol from testosterone, as well as the formation of estriol and estrone from other androgen precursors in vitro (Azcoitia et al., 2011). CYP19A1 can be visualized in rat, monkey, and human brain with the radiolabeled inhibitor \([^{11}C]\)-vorozole (Biegon et al., 2010; Takahashi et al., 2006).

We are currently unaware of any characterized substrates or inhibitors of CYP2D6 that could be used in PET imaging studies of brain CYP2D6, creating the need for surrogate measures of brain CYP2D6 activity. One potential technique for imaging brain CYP2D6 would be to use radioligand displacement studies directed at the functional effect of a CYP2D6-mediated metabolite, such as the CYP2D6 probe substrate dextromethorphan metabolism to dextrorphan. Dextromethorphan blocks excitatory n-methyl-d-aspartate (NMDA) receptors in vitro; dextrorphan also binds to NMDA but with greater affinity than dextromethorphan (Church et al., 1994; Parsons et al., 1995). In monkeys trained to discriminate phenylcyclidine (an NMDA receptor antagonist) from saline, dextrorphan was a greater reinforcer than dextromethorphan indicative of greater NMDA receptor
activation (Nicholson et al, 1999). Furthermore, the positive reinforcing effects of dextromethorphan were reduced in individuals pretreated with the CYP2D6 inhibitor quinidine, suggesting CYP2D6 metabolism to dextrorphan may be responsible for dextromethorphan response (Zawertailo et al, 2010). Assessing dextrorphan-induced NMDA receptor antagonism is one potential technique for elucidating brain CYP2D activity in vivo. While there is no agreed upon PET radiotracer for labelling NMDA receptors, there are numerous candidates (e.g. fluoropiperidine derivatives) that display high selectivity and brain uptake (Kassenbrock et al, 2016). If brain CYP2D6 enzyme activity is increased in smokers (or individuals administered nicotine), the brain CYP2D6 metabolism of dextromethorphan to dextrorphan would be increased along with the resulting dextrorphan-induced NMDA receptor antagonism, presenting as a decrease in CNS NMDA receptor binding and radiolabelling by the PET tracer.

Lastly, there has been progress made towards using functional magnetic resonance imaging (fMRI) techniques to quantify brain perfusion as an indicator of CYP2D6 genotype. Brain perfusion at rest is a well-characterized index of cerebral activity; variation in resting perfusion is associated with specific personality traits, as well as genetic polymorphisms in proteins associated with these traits (e.g. serotonin transporter) (Kirchheiner et al, 2011; Rao et al, 2007; Shulman et al, 1997). CYP2D6 genotype may associate with personality phenotypes, due to the metabolism of endogenous compounds (demonstrated in vitro) such as the neurotransmitter dopamine and the endocannabinoid anandamide (Hiroi et al, 1998b; Snider et al, 2008). Relative to CYP2D6 EMs, CYP2D6 PMs had 15% higher perfusion levels in the thalamus; fMRI detection of brain perfusion provided relatively successful prediction of individuals as CYP2D6 UMs (sensitivity and specificity ranging from 85–87%) and CYP2D6 PMs (71–79%) (Kirchheiner et al, 2011; Napolitano et al, 2017). Brain perfusion in individuals exposed to xenobiotic inducers of brain CYP2D6 (e.g. nicotine or ethanol) could be assessed as a proxy of brain CYP2D6 activity, where decreased brain perfusion would be indicative of increased brain CYP2D6 activity. However, this technique would also be sensitive to secondary changes associated with those xenobiotic compounds; for example, smokers show decreased brain perfusion in brain regions associated with maintenance of addictive disorders (Durazzo et al, 2015). Despite this, the effect of nicotine alone (e.g. through nicotine replacement therapy, or electronic-cigarettes) on brain perfusion is
unknown. Active investigation is underway to determine the optimal imaging technique for translation of our preclinical findings on inducible brain CYP2D to humans.

5.5.3 Therapeutic targeting of brain CYP2D6 in humans

The potential to inhibit or induce brain CYP2D6 with, or independently of, liver CYP2D6 in humans for therapeutic purposes is an interesting extension of our brain metabolism research. In regards to this thesis specifically, targeted inhibition or induction of the brain CYP2D6 metabolism of opioids like codeine or oxycodone could be used to 1) modulate acute analgesia, for example by inhibiting brain CYP2D6 in CYP2D6 UMs prior to oxycodone treatment to increase analgesic response, or to 2) decrease the rate of development of tolerance, for example by inhibiting brain CYP2D6 in CYP2D6 UMs prior to codeine treatment to reduce acute analgesic response.

Increasing brain CYP2D6 activity in humans may be of therapeutic use in protecting against neurotoxicity. As outlined in section 1.4.4.3.1, individuals with Parkinson’s disease have 40% lower brain CYP2D6 levels than age-matched controls, and CYP2D6 PMs are at greater risk for developing Parkinson’s disease (Elbaz et al, 2004; Mann et al, 2012; Mann and Tyndale, 2010). As many neurotoxins are inactivated by CYP2D6 (e.g. MPTP, β-carbolines), brain CYP2D6 activity may function to protect against neurotoxicity, as seen in Parkinson’s disease (Miksys and Tyndale, 2006; Zanger et al, 2004). In support of this, smokers are at a lower risk for Parkinson’s disease which may be due, in part, to greater inactivation of neurotoxins via increased brain CYP2D6 levels in smokers (mentioned in introduction section 1.4.4.3.1) (Alves et al, 2004; Miksys and Tyndale, 2006).

The most direct method for inducing brain CYP2D6 specifically, as utilized in the animal model paradigms in this study, would be to use xenobiotic inducers of brain CYP activity. As discussed in section 1.4.4.2, while hepatic CYP2D6 levels in humans are primarily regulated by genetics, brain CYP2D6 activity may be altered by exposure to select xenobiotics. Smokers and alcoholics have higher CYP2D6 levels in the brain, while nicotine and ethanol increase rat brain CYP2D expression and dose-dependently increase monkey brain CYP2D expression (Mann et al, 2008; Miksys et al, 2002; Miksys and Tyndale, 2004; Miller et al, 2014; Warner and Gustafsson, 1994; Yue et al, 2008). However, the individual influences of nicotine and ethanol on human brain CYP2D6 in vivo have yet to be characterized, including associated nuances, such as the time-
course of effect, the dose dependency of effect, the functional outcome of effect on drug metabolism and response, etc.

Conversely, gene therapies, like the use of microRNA (miRNA), could be used for brain specific down-regulation of CYP2D activity. miRNA are short non-coding RNA strands complementary to targeted mRNAs, that function as components of the RNA-induced silencing complex leading to translational inhibition or degradation of those specific mRNA (Esteller, 2011). As briefly mentioned in 1.4.4.2, exogenous testosterone administration decreased CYP2D levels in rat brain; testosterone increased levels of brain-specific miRNA (miR-101 and miR-128-2) in human neurons (SH-SY5Y) and astrocytes (U251) but not hepatocytes (HepG2) in vitro, while overexpression of those miRNA decreased CYP2D mRNA (Li et al, 2015a). Testosterone decreased levels of the CYP2D metabolite O-desmethyltramadol in rat brain, but not plasma, after i.p. tramadol administration, and decreased in tramadol analgesia, suggesting testosterone was inhibiting brain CYP2D activity in vivo, likely by upregulation of miRNA (Li et al, 2015a).

Delivering miRNA to the brain is a challenge, as the BBB hinders accumulation of active compounds in the CNS and therefore limits transfection efficiency. The two strategies used most commonly are miRNA mimics to restore suppressed miRNA levels, or anti-miRNA to repress overactive miRNA function (van Rooij and Kauppinen, 2014). In general, gene therapies using RNA interference (like miRNA) lack stability due to degradation, therefore delivery systems are used to both protect miRNA, and assist their entrance into the CNS (Chen et al, 2015). Some examples include viral vectors, lipid-based carriers, gold nanoparticles, polymer-based carriers, carbon-based carriers, and magnetic resonance-guided focused ultrasound (Wen, 2016). The delivery of miRNA specific to brain CYP2D6 in humans could be utilized to therapeutically inhibit brain CYP2D6, therefore increasing the brain levels of CYP2D-substrate parent compounds and therapeutic effect or preventing the formation of active toxic metabolite(s). Specific to this thesis, inhibiting brain CYP2D6 could function to regulate short and long-term effects to opioid compounds, for example decreasing acute codeine analgesia and decreasing the rate of codeine tolerance.
5.5.4 The mechanism of inducible brain-specific CYP2D

The neural mechanism(s) behind the brain specific induction of CYP2D by nicotine pretreatment is currently unknown. Nicotine treatment increased brain CYP2D expression in rats and monkeys, neither of which were associated with changes in brain CYP2D mRNA levels, or changes in hepatic CYP2D enzyme activity and mRNA levels (Mann et al., 2008; Miller et al., 2014; Yue et al., 2008). Because nicotine did not increase brain CYP2D mRNA levels, this indicated that CYP2D induction likely occurs via non-transcriptional events, such as increasing translational efficiency (decreased splicing or increased translation), increasing enzyme stability, or decrease enzyme degradation.

One potential mechanism behind the induction of brain CYP2D by nicotine is alteration of the ubiquitin-mediated protein degradation of CYP2D in the brain specifically. The binding of ubiquitin targets proteins for proteasome degradation and fragmentation, where alterations in this pathway may produce functional changes in enzyme expression (Kane et al., 2004). For example, the induction of rat liver CYP2E1 by ethanol is accompanied by decreased CYP2E1 protein ubiquitination (Roberts et al., 1995). Ethanol induced CYP2E1 in rat liver microsomes without changing mRNA levels; less radiolabeled CYP2E1 activity was lost from ethanol-induced microsomes (50%) than control microsomes (93%) in the first 48 hr (Roberts et al., 1995). After inducing protein degradation with carbon tetrachloride, there were fewer immunolabeled ubiquitin-CYP2E1 complexes in liver microsomes from rats treated with ethanol than in control livers microsomes (Roberts et al., 1995). Nicotine also impacts ubiquitination-related proteins in rat brain, though this is in a region-specific manner. Ubiquitin, ubiquitin-conjugating enzymes, proteasomal subunits, and ubiquitin-chaperone proteins were all upregulated by nicotine in the prefrontal cortex, but downregulated in the medial basal hypothalamus (Kane et al., 2004). The paradigm of short-term nicotine pretreatment used in this thesis may be preferentially down-regulating ubiquitin-related proteins in the brain regions associated with greatest increases in CYP2D protein activity (cerebellum, hippocampus, and striatum) (Yue et al., 2008). A study similar to that which identified ethanol induction of CYP2E1 could be designed to look at this directly, with brain membranes from rats pretreated with nicotine compared to vehicle, as well as antibodies raised against rat CYP2D isoforms and ubiquitin.
5.6 Conclusions

Our findings contribute to the existing literature on brain CYP metabolism as a novel source of variability through brain specific drug metabolism and resulting drug response. Using paradigms developed in our laboratory to inhibit (by direct intracerebroventricular injection of propranolol and propafenone) and induce (by short-term pretreatment with nicotine) rat CYP2D enzyme specifically in the brain, we investigated the impact of brain CYP2D metabolism in vivo on codeine and oxycodone central metabolism and resulting effect. We found that inhibiting brain CYP2D in rats decreased, while inducing brain CYP2D increased, brain morphine levels from codeine and resulting codeine analgesia. We further found that the increase in acute codeine analgesia after inducing brain CYP2D resulted in a more rapid rate of onset of tolerance across repeated codeine administration. The rate of tolerance, or decrease in analgesia per dose, correlated to the acute analgesic response following the first dose of codeine, which itself was dependent on codeine dose or level of brain CYP2D activity. Lastly, we found that, in contrast to codeine analgesia, oxycodone analgesia was increased by inhibiting brain CYP2D, and was decreased by inducing brain CYP2D. Brain drug levels were assessed through in vivo microdialysis, and brain oxycodone levels were increased after inhibiting brain CYP2D, while brain oxymorphone levels were increased after inducing brain CYP2D, indicating that brain oxycodone levels were primarily responsible for oxycodone analgesia.

Opioid analgesics, while effective for the intended treatment of pain, are often misused and abused resulting in opioid-related morbidity and mortality. Interindividual variation in CNS-acting drug response, specifically in opioid-induced pain relief, and risk for dependence, is not fully understood. The brain CYP metabolism of opioids, as we have explicitly shown for brain CYP2D, could explain some of this variation, and add to the known risk factors for failure of opioid therapeutic effect, as well as consequences of chronic use like tolerance, transition to abuse, and overdose.

The tissue-specific regulation of CYP2D may have a unique impact on drug metabolism and response. Smokers, similar to rats and monkeys pretreated with nicotine, have greater CYP2D levels in the brain, but not liver, compared to non-smokers; smokers may therefore experience altered CNS-acting drug metabolism and drug response (e.g. increased activation of codeine to
morphine, or increased metabolism, and therefore removal, of oxycodone to oxymorphone), not
detected in peripheral pharmacokinetics. Remaining questions regarding the role of brain CYP2D
in opioid metabolism include: 1) the impact of brain CYP2D metabolism of opioids on abuse
liability, 2) the mechanism(s) behind the induction/regulation of brain CYP2D in animals and brain
CYP2D6 in humans, 3) the development of useful clinical measures or imaging techniques for
predicting and detecting brain CYP2D6 expression, function, and impact in humans, and 4)
whether inhibition or induction of brain CYP2D6 in humans (either through targeted therapeutics
or drug-drug interactions) produces similar functional outcomes as those seen in rats in vivo.
6. References


Loyd DR, Murphy AZ (2009). The role of the periaqueductal gray in the modulation of pain in males and females: are the anatomy and physiology really that different? *Neural Plast* 2009: 462879.


Abstract
The functional role of cytochrome P450 (CYP) enzymes in the brain is an exciting and evolving field of research. CYPs are present and active in the brain, with heterogeneous patterns of expression, activity, and sensitivity to modulation across cell types, regions, and species. Despite total brain CYP expression being a fraction of hepatic CYP expression, the expanding literature of in vitro and in vivo experiments has provided evidence that brain CYPs can impact acute and chronic drug response, susceptibility to damage by neurotoxins, and are associated with altered personality, behaviour, and risk of neurological disease. They may also play a role in endogenous neurotransmitter and neurosteroid homeostasis. This review goes through the characterization of brain CYPs across species, the patterns of susceptibility of brain CYPs to exogenous induction, and recent preclinical evidence of the potential role of brain CYPs in vivo (e.g. CYP2D), along with the development of experiment paradigms that allow modulation of brain CYP activity without affecting CYP activity in the liver. Understanding brain CYP function, and changes therein, may provide unique strategies for the development of CNS-acting therapeutics metabolized locally in the brain, as well as therapeutics to target brain CYPs directly.
1. Introduction to brain CYP enzymes

Cytochrome P450 enzymes (CYPs) are a major source of variability in pharmacokinetics of exogenous and endogenous compounds. CYPs are regulated in a variety of ways including genetic variation, and xenobiotic and endogenous (cytokines and hormones) induction and repression (Zanger and Schwab, 2013). While drug metabolism occurs predominantly in the liver, CYP-mediated drug metabolism within the brain has emerged as another source of variation in drug response (Miksys and Tyndale, 2013). Total brain CYP content is low relative to liver (approximately 0.5–2%) and is therefore unlikely to influence systemic drug and metabolite levels of CYP substrates (Hedlund et al., 2001). However, brain CYP-mediated metabolism of centrally acting compounds can impact local brain levels and resulting therapeutic effects, independent of peripheral metabolism and systemic drug levels. The potential impact of variable brain CYP activity is substantive, with demonstrated effects on behaviour, neurotoxicity, and drug response (e.g. analgesics, anesthetics, antidepressants, antipsychotics) (Miksys and Tyndale, 2013; Toselli et al., 2016; Wang et al., 2014). The focus of this review will be the evolving understanding of the functional impact of brain CYP families 1–4, including newly developed animal models of increased and decreased brain CYP activity, as well as new findings in brain CYP-mediated metabolism of exogenous and endogenous compounds. Outside of this scope there have been substantial advancements in the research areas of additional CYPs within the brain (e.g. CYP17, CYP27, and CYP46), however we have focused on CYP1–4 due to their role in the metabolism of a broad range of clinically relevant central nervous system (CNS)-acting compounds, and the contrasting regulation of these CYPs in brain relative to that in liver.

2. Experimental in vivo tools for investigating the role of brain CYPs

Early evidence of CYP activity in the brain came in the 1970s (Fishman et al., 1976; Paul et al., 1977; Sasame et al., 1977), which was followed by research into subcellular localization, activity, and substrate specificity compared to liver CYP enzymes (Miksys and Tyndale, 2004). Substantial interest exists in brain CYPs due to the potentially unique impact of CYP-mediated metabolism within the brain (e.g. on the blood-brain barrier (BBB)). This is despite CYP expression being much lower in the brain than in liver, and also no prior knowledge of the existence of sufficient levels and locations of cofactors and coenzymes required for CYP-mediated metabolism (Hedlund et al., 2001). Early detection of CYP activity ex vivo was limited due to low enzyme levels and
rapid degradation; for example, nearly 50% of brain CYP2D activity was lost within a week of freezing (Tyndale et al, 1999). Nevertheless, reactions catalyzed by CYPs ex vivo have occurred using brain membranes; for example, the CYP2D oxidation of dextromethorphan to dextrophan by human and rat brain microsomes (Tyndale et al, 1999; Voirol et al, 2000). Animal models have been fundamental in elucidating the impact of brain CYP-mediated metabolism on centrally-acting drug metabolism and response. The ability to measure drug levels directly in the brain (e.g. by in vivo microdialysis), specific and selective manipulation of brain CYP activity with pharmacologic inhibitors and inducers (modeled in Figure 1), molecular inhibition techniques, and transgenic animal models have provided in vivo evidence of the impact of CYPs expressed in the brain.

2.1. Models of decreased brain CYP activity
One approach to selective brain inhibition is direct intracerebroventricular (ICV) administration of CYP inhibitors which have no impact on hepatic metabolism confirmed in vivo and ex vivo. The unique advantage of mechanism-based inhibitors is that they require CYP-mediated activation prior to their metabolites binding to, and inactivating, the same enzyme; binding of the metabolite to the enzyme demonstrates that the enzyme within the brain is functional in situ (Miksys and Tyndale, 2009). The in vivo activity of brain CYPs was first demonstrated in rats through the ICV injection of the CYP2B mechanism-based inhibitors 8-methoxypsoralen and C8-xanthate (Miksys and Tyndale, 2009). [3H]-8-methoxypsoralen was injected into rat frontal cortex where it was enzymatically activated by, and subsequently radiolabelled, CYP2B (Miksys and Tyndale, 2009). ICV injection of C8-xanthate into one side of the frontal cortex inhibited brain CYP2B on that side alone, as indicated by a reduction in CYP2B radiolabelling by [3H]-8-methoxypsoralen and therefore a decrease in CYP2B activity (Miksys and Tyndale, 2009). Further proof of the specific enzyme involved was provided by the immunoprecipitation of the radiolabelled protein with antibodies to CYP2B (Miksys and Tyndale, 2009). This demonstrated both the in vivo activity of rat brain CYP2B, and specificity of binding of the mechanism-based inhibitors. Similarly, propranolol is a mechanism-based inhibitor of CYP2D; propranolol is first hydroxylated by CYP2D to a reactive metabolite that binds the active site of the enzyme, inactivating it (Narimatsu et al, 2001). ICV propranolol inhibits brain, but not liver, CYP2D activity in rats, as determined by decreased ex vivo oxidation of the CYP2D substrate codeine by rat brain, but not hepatic, membranes (Zhou et al, 2013).
Figure 1: Model of the expected outcome of altered in vivo brain CYP2D activity on CNS-acting drug response, where the CYP2D-formed metabolite is more active than the parent compound. Control brain CYP2D metabolism of parent drug to metabolite results in baseline drug effect (a). Inhibiting brain CYP2D specifically (e.g. ICV administration of a mechanism-based inhibitor; INH) reduces metabolite production in brain, and decreases the resulting drug effect (b). In contrast, brain-specific induction of CYP2D (e.g. chronic nicotine administration) increases metabolite production in brain, and increases the resulting drug effect (c). ICV inhibitor delivery subsequent to brain CYP2D induction attenuates both the increase in metabolite production in brain and the increased drug effect (d).

Gene silencing through RNA interference has emerged as a promising technique for reducing CYP protein levels in vivo, although its use is limited due to difficulty resulting from inadequate transport of nucleic acids across CNS membranes (Pardridge, 2007). MicroRNAs (miRNAs) function as RNA-induced silencing complex guides, mediating translational inhibition and degradation of targeted genes and proteins (Esteller, 2011). Brain-enriched miRNA has been implicated in organ-specific regulation of brain CYP2D; exogenous testosterone (given
subcutaneously) decreases rat brain, but not liver, CYP2D levels (Li et al., 2015a). In culture, testosterone increases brain-specific miR-101 and miR-128-2 in human brain-derived cell lines (SH-SY5Y and U251); overexpression of miR-101 and miR-128-2 decreased CYP2D mRNA levels in these cells (Li et al., 2015a).

More general approaches include whole brain-CYP knockout animal models (e.g. brain neuron-specific deletion of the Cpr gene encoding cytochrome-P450 reductase, required for CYP function; brain-Cpr-null) (Conroy et al., 2010). Various general CYP inhibitors (e.g. miconazole and fluconazole) can also be used to assess metabolism by brain CYPs (Hough et al., 2011).

2.2. Models of increased brain CYP activity
CYPs in the brain appear to be especially sensitive to induction following xenobiotic exposure. Brain CYP expression can be altered in addition to, or independent of, an effect on liver CYP activity (e.g. phenobarbital increases monkey CYP2B expression in both the liver and brain, but increases CYP2E expression in brain, but not liver (Lee et al., 2006a; Lee et al., 2006c)) (Table 1). Chronic nicotine treatment induces rat and monkey brain, but not liver, CYP2B and CYP2D enzymes, but induces CYP2E enzyme in both the brain and liver (Ferguson et al., 2011; Ferguson et al., 2012, 2013; Howard et al., 2001; Joshi and Tyndale, 2006a, b; Khokhar et al., 2010; Lee et al., 2006b; Lee et al., 2006d; Mann et al., 2008; Micu et al., 2003; Miksys et al., 2000b; Miller et al., 2014; Yue et al., 2008).
Table 1. Summary of the effects of nicotine and ethanol on liver and brain CYP expression across species.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Isozyme</th>
<th>Liver</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rat</td>
<td>Monkey</td>
</tr>
<tr>
<td>Nicotine / Smoking</td>
<td>CYP2B</td>
<td>– a,b</td>
<td>– c,d</td>
</tr>
<tr>
<td></td>
<td>CYP2D</td>
<td>– a,l</td>
<td>– j,k</td>
</tr>
<tr>
<td></td>
<td>CYP2E</td>
<td>↑ a,n,o</td>
<td>↑ p</td>
</tr>
<tr>
<td>Ethanol / Alcoholism</td>
<td>CYP2B</td>
<td>↑ a,0</td>
<td>↑ c</td>
</tr>
<tr>
<td></td>
<td>CYP2D</td>
<td>– a</td>
<td>– k</td>
</tr>
<tr>
<td></td>
<td>CYP2E</td>
<td>↑ a,n,0</td>
<td>↑ p,x</td>
</tr>
</tbody>
</table>

Similarly, ethanol has distinct effects on brain and liver CYP expression. In rats, ethanol increases CYP2D levels in the brain, CYP2B levels in the liver, and CYP2E levels in both brain and liver (Howard et al., 2003; Schoedel et al., 2001; Warner and Gustafsson, 1994; Yue et al., 2009). In monkeys, ethanol induced brain, but not liver, CYP2D while inducing brain and liver CYP2B and CYP2E levels (Ferguson et al., 2011; Ferguson et al., 2012, 2013; Miller et al., 2014). In human autopsy tissue, prior smoking (chronic nicotine exposure) and alcohol (ethanol) consumption have similar effects on brain and liver CYP expression as chronic nicotine and alcohol in rats and monkeys. Smokers and alcoholics have higher brain levels of CYP2B6 and CYP2E1 compared to non-smoking non-alcoholics, however due to high co-consumption, it is unknown if this is a result of independent effects (or other variables) of the two compounds (Howard et al., 2003; Miksys et al., 2003; Zhong et al., 2012). Smokers have higher hepatic CYP2E1, with no change in hepatic CYP2B6 protein levels or activity, compared to non-smokers, while alcoholics (or drinkers) have increased hepatic CYP2B6 and CYP2E1 activity (Benowitz et al., 2003; Hesse et al., 2004; Tsutsumi et al., 1989). Human brain CYP2D6 levels are higher in smokers and alcoholics, compared to non-smokers and non-alcoholics, with no change in liver CYP2D activity (Mann et al., 2008; Miksys et al., 2002; Miksys and Tyndale, 2004). The mechanisms and patterns of brain CYP induction by xenobiotic exposure are not well characterized; details on the region-specificity, and potential mechanisms, of xenobiotic induction of brain CYPs are discussed later in the paper. The sensitivity of brain CYPs to induction has been utilized in vivo in proof-of-concept studies of
the role of brain CYP-mediated metabolism of exogenous compounds (discussed in the next section).

Humanized transgenic animal models of overexpression of brain CYPs are another tool for examining metabolism by brain CYPs. A human CYP2D6 transgenic (Tg-2D6) mouse model was generated with human CYP2D6 protein expressed in the liver and brain; compared to wild-type, Tg-2D6 mice have greater basal serotonin levels (which can be formed in vitro by CYP2D) and lower harmaline induced tremors (metabolized by CYP2D), suggestive of increased brain CYP2D activity (Cheng et al., 2013).

3. Evidence of a functional role for brain CYPs
The development of preclinical animal models, whereby brain-specific CYP expression and activity can be altered, facilitates investigation into the impact of brain CYP-mediated metabolism in drug efficacy, neurotoxicity, and endogenous compound metabolism. Using animals to model the potential variation in human brain CYP-mediated metabolism has provided insight into behavioural consequences of elevated and reduced brain CYP expression and activity in vivo.

3.1. The role of brain CYPs in response to exogenous compounds
The response to centrally acting drugs is variable and not always predicted by plasma drug levels. CNS-drug response may reflect brain drug levels, modulated, at least in part, by local metabolism by CYPs. Propofol is a sedative-anesthetic that is inactivated by CYP2B hydroxylation (Court et al., 2001). Selectively inhibiting (with ICV C8-xanthate) and inducing (with short-term peripheral injections of nicotine) rat brain CYP2B yielded increased and decreased, respectively, brain propofol concentrations and propofol responses (measured by sleep duration) (Khokhar and Tyndale, 2011). The effects of nicotine pretreatment were reversed by ICV C8-xanthate pretreatment, suggesting that the pretreatments were acting by similar mechanisms of action, namely via manipulation of brain CYP2B (through induction and inhibition, respectively) (Khokhar and Tyndale, 2011). Propofol-induced sleep time correlated with brain, but not plasma, propofol levels (Khokhar and Tyndale, 2011). This pretreatment paradigm allows alteration of brain CYP2B activity while maintaining normal CYP2B function in the liver, as tested both in vivo (via systemic drug and metabolite levels) and ex vivo (Khokhar and Tyndale, 2011).
Nicotine, an inducer of rat brain CYP2B when given as a short-term pretreatment, is metabolized by brain CYP2B in rats (versus CYP2A which is the main hepatic nicotine-metabolizing enzyme in mice and humans) (Garcia et al, 2015). In similarly designed studies, inhibiting and inducing rat-brain CYP2B resulted in an increase and decrease, respectively, of brain, but not plasma, nicotine levels (Garcia et al, 2015; Garcia et al, 2016). Brain nicotine levels were assessed over time by microdialysis, demonstrating the usefulness of in vivo brain microdialysis in assessing changing drug and metabolite brain levels in rat behavioural models of altered brain CYP-mediated metabolism. Inhibiting brain CYP2B (with ICV C8-xanthate, resulting in higher brain nicotine levels) resulted in a leftward shift of the dose-acquisition curve for nicotine self-administration; a greater percentage of animals reached acquisition-criteria at lower nicotine doses (Garcia et al, 2015). It also resulted in greater responding when tested on a progressive-ratio, thought to reflect increased nicotine-reinforcement (Garcia et al, 2015). Human smokers who are genetically CYP2B6 slow metabolizers report greater nicotine craving scores during abstinence and are more likely to relapse during nicotine withdrawal, likely due to neuroadaptations in response to higher brain nicotine levels, suggesting a potential role for brain CYP2B6 in nicotine metabolism and long-term effects in smokers (Lerman et al, 2002).

Almost all oral opioid drugs (e.g. hydrocodone) have activity at the mu-opioid receptor, and are enzymatically activated by CYP2D to more potent metabolites (e.g. hydromorphone) (Smith, 2011). CYP2D6 metabolizes the prodrug codeine to morphine, a step required for codeine-induced analgesia; human CYP2D6 poor metabolizers (no CYP2D6 activity), and individuals given CYP2D6 inhibitors systemically, produce no/less morphine, and experience no/less analgesia, from codeine (Chen et al, 1991b; Sindrup et al, 1992). Inhibiting rat brain, but not liver, CYP2D (with ICV propranolol) decreased brain, but not plasma, morphine levels and decreased codeine analgesia after peripheral codeine administration (Zhou et al, 2013). Further, inducing rat brain CYP2D with short term nicotine pretreatments increased brain, but not plasma, morphine levels and increased codeine analgesia; the change in codeine analgesia after nicotine pretreatment induction of brain CYP2D was subsequently reversed by inhibiting brain CYP2D with ICV propranolol mechanism-based inhibitor pretreatment (Figure 2) (McMillan and Tyndale, 2015). Codeine analgesia correlated with brain, but not plasma, morphine levels after codeine
administration (McMillan and Tyndale, 2015). Neither inhibiting nor inducing brain CYP2D had any effect on drug levels or response following systemic administration of morphine (instead of codeine); morphine is not a CYP2D substrate (McMillan and Tyndale, 2015). The selective impact of these manipulations on codeine, but not morphine, metabolism and response, indicated that the pretreatments acted through altering rat brain CYP2D metabolism, rather than off-site actions of the pretreatments (McMillan and Tyndale, 2015). This acute effect of brain metabolism was replicated with the analgesic tramadol, another opioid drug metabolically activated by CYP2D. Selective rat brain CYP2D inhibition (with ICV propranolol) decreased brain tramadol metabolism, measured as a decrease in brain levels of the CYP2D metabolite O-desmethyltramadol (O-DSMT) and a prolongation of tramadol's elimination t1/2 (Wang et al, 2015). Inducing rat brain CYP2D with nicotine increased tramadol analgesia, O-DSMT brain concentration, and shortened the T_max of tramadol effect (Wang et al, 2015). Inhibiting and inducing rat brain CYP2D did not alter the peripheral pharmacokinetics of tramadol in the plasma, measured as the relative extent of conversion of tramadol to O-DSMT (Wang et al, 2015). Furthermore, the increase in tramadol analgesia after nicotine pretreatment was attenuated by inhibiting brain CYP2D (Wang et al, 2015). Beyond acute response, inducing rat brain CYP2D with nicotine increased the rate of tolerance to codeine's analgesic effects; inducing brain CYP2D increased initial codeine analgesia, and resulted in a more rapid decrease in analgesia across daily codeine administration (McMillan and Tyndale, 2017b). The rate of tolerance (decrease in peak analgesia per day) correlated to initial response, suggesting that changes in initial codeine analgesia (e.g. via inhibiting or inducing brain CYP2D metabolism to morphine) would predict changes in tolerance (McMillan and Tyndale, 2017b). Thus, people with increased brain CYP2D activity (genetically or as a result of CNS CYP2D induction) may experience greater acute opioid-induced analgesia from codeine or tramadol due to increased central opioid activation, as well as a more rapid onset of analgesic tolerance, suggesting a potentially increased impetus for dose escalation. These data also suggest that brain CYP2D may contribute to metabolism and analgesia of other opioids (e.g. hydrocodone, oxycodone), as well as to other consequences of opioid use (e.g. side-effects, tolerance, dependence, and withdrawal).
Figure 2: Manipulating in vivo rat brain CYP2D activity alters central metabolism of codeine to morphine and resulting codeine analgesia. Rat brain CYP2D was inhibited by ICV pretreatment with propranolol (PL, CYP2D mechanism-based inhibitor given 24 h ahead of codeine), induced with chronic sub-cutaneous pretreatment with nicotine, or induced and subsequently inhibited prior to codeine administration (illustrated in a). Codeine is a prodrug that exhibits little-to-no activity at the μ-opioid receptor and must first be activated by CYP2D to morphine to produce analgesia. Compared to control animals (grey lines and bars; no inhibitor or inducer), inhibiting brain CYP2D (grey lines and bars with red border) decreased analgesia at multiple time points after codeine (b: %MPE, maximum possible effect) presenting as a decrease in area under the analgesia-time curve surrounding peak analgesia (c: AUC0–30), and decreased brain morphine levels after codeine administration (d). Inducing brain CYP2D (purple bars and lines) increased %MPE (b), analgesic AUC0–30 (c), and brain morphine levels (d) after codeine administration. Inhibiting the induced brain CYP2D (co-pretreatment with both brain CYP2D inhibitor and inducer; purple bars and lines with red border) attenuated the induced %MPE (b), analgesic AUC0–30 (c), and brain morphine levels (d) after codeine administration. The full study is described in McMillan and Tyndale (2015). *p < 0.05 vs control; *p < 0.05, **p < 0.01 vs inhibitor plus inducer group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Neuronal CYP epoxygenases may also play a significant, although indirect, role in exogenous and endogenous produced analgesia. Analgesia in mice, mediated by opioid-receptor activation, requires CYP-mediated epoxygenation of arachidonic acid (AA) to epoxyeicosatrienoic acids (EETs), which themselves may activate analgesic circuits via unknown mechanisms (Conroy et al., 2010; Spector, 2009). The production of EETs from AA is mediated by numerous CYP isoforms, namely human CYP2C and CYP2J, as well as the orphan enzyme human CYP4X1 (Iliff et al., 2010; Stark et al., 2008). Decreased mouse brain CYP activity, via transgenic knockout (brain-Cpr-null) or pharmacologic inhibition (miconazole and fluconazole), attenuated morphine (opioid) and improgan (non-opioid) analgesia (Conroy et al., 2010; Hough et al., 2011). Morphine-induced side effects (respiratory depression, locomotor stimulation, and inhibition of intestinal motility) were not affected by decreased brain CYP epoxygenase activity, suggesting that the mechanism of these side-effects is dissociated from that of analgesia (Hough et al., 2014a; Hough et al., 2015). A similar reduction of morphine analgesia was observed in mice treated with selective CYP epoxygenase inhibitors (MW06-25 and MS-PPOH) (Conroy et al., 2010).

3.2. The protective role of brain CYP-mediated metabolism in neurotoxicity
A large number of central neurotoxins (e.g. organophosphate pesticides, MPTP, TIQ, paraquat and harmaline) are metabolized by CYP enzymes (Buratti et al., 2003; Miksys and Tyndale, 2006). Peripheral metabolism phenotypes have been used as predictors of neurotoxic risk; however, target-site metabolism by brain CYPs may be a novel source of variation in individual susceptibility to neurotoxic insult. Parkinson's disease (PD) is a progressive neurodegenerative disorder with an unknown etiology; both genetic variation and environmental exposure to neurotoxins contribute to PD (Elbaz et al., 2004). Individuals with a CYP2D6 poor metabolizer phenotype (no CYP2D6 activity) are at a greater risk for PD, which increases with exposure to neurotoxins (Elbaz et al., 2004). PD patients have approximately 40% lower brain CYP2D6 levels than age-matched genotype-matched controls, suggesting that low brain CYP2D6 may be a risk factor for PD (Mann et al., 2012). The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its toxic metabolite 1-methyl-4-phenylpyridinium (MPP+) are metabolically inactivated by CYP2D6; both MPTP and MPP+ are used in experimental animal models of PD (Mann and Tyndale, 2010). Overexpression of CYP2D6 in cultured rat adrenal medulla (PC12) cells is protective against neurotoxicity from MPP+, consistent with increased MPTP and
MPP\(^*\) neurotoxicity following inhibition of CYP2D6 in cultured human neuroblastoma (SH-SY5Y) cells (Mann and Tyndale, 2010; Matoh et al, 2003). This suggests that variation in the activity or expression of CYP2D6 in the brain may affect neurotoxin metabolic inactivation and degree of neurotoxicity. Tobacco smokers are at lower risk for PD, and nicotine has been found to be neuroprotective in several neurotoxin-induced animal models of the disease (Alves et al, 2004; Quik et al, 2009). Chronic nicotine treatment protects against MPTP-induced nigrostriatal damage in mice, a brain region predominantly affected in PD (Janson et al, 1992). Nicotine induction of brain, but not liver, CYP2D, among other CYP enzymes, may contribute to the protection against PD in smokers by increasing metabolic inactivation of neurotoxins (Miksys and Tyndale, 2006).

Chlorpyrifos (CP) is an organophosphate insecticide that is metabolically activated by CYP2B to the chlorpyrifos-oxon (CPO), a metabolite that inhibits acetylcholinesterase (AChE) causing chlorpyrifos neurotoxicity (Sultatos, 1994). CPO is quickly deactivated by esterases in the liver and circulating blood, suggesting that peripherally formed CPO may not reach the brain (Costa et al, 1990); however, central CPO production by brain CYP metabolic activation may contribute to CP-induced neurotoxicity. In a rat-model of CP-induced neurotoxicity, inhibiting rat brain CYP2B (with ICV C8-xanthate) increased brain CP levels, decreased brain CPO levels, reduced CPO-mediated AChE inhibition in brain, and reduced the severity of CPO-induced hypothermia and neurotoxicity (assessed with a battery of behavioural tests) (Khokhar and Tyndale, 2012). Consistent with the hypothesis that CP neurotoxicity results from brain CYP2B activation to CPO, there were no differences in plasma CP levels, plasma CPO levels, and in peripheral AChE inhibition following inhibition of brain CYP2B (Khokhar and Tyndale, 2012). CP is a long-lasting neurotoxin, remaining in the body for prolonged periods of time. Even days after CP administration, deficits in behaviour were reduced by subsequent ICV C8-xanthate (brain-specific CYP2B inhibition) as well as by intra-peritoneal C8-xanthate, given peripherally to model human consumption of an oral therapeutic inhibitor (shown to inhibit brain and hepatic CYP2B) (Khokhar and Tyndale, 2014). Inhibiting brain metabolism could be a potential therapeutic approach for reducing the long-lasting levels of CP in the body, and resulting CP toxicity (Khokhar and Tyndale, 2012, 2014).
Individual risk of experiencing side-effects with acute or chronic antipsychotic treatment varies substantially. Haloperidol is an antipsychotic drug and CYP2D substrate that is associated with severe extra pyramidal symptoms (EPS), including tardive dyskinesia (Lockwood and Remington, 2015). Catalepsy is used as a model of acute haloperidol induced EPS; selectively inhibiting rat brain, not hepatic, CYP2D, reduced catalepsy induced by acute haloperidol, while inducing brain CYP2D increased catalepsy (Miksys et al, 2017). This suggests that brain CYP2D was responsible for metabolizing haloperidol to a neurotoxic metabolite and that reducing CYP2D activity in the brain was neuroprotective for this acute haloperidol induced toxicity. While few reports have studied the effects, acute or chronic, of smoking on EPS, there is some evidence that antipsychotic-induced side-effects are more prevalent in smokers (Wagner et al, 1988). Increased brain CYP2D levels in smokers may contribute to a greater risk for acute EPS from antipsychotic therapy; this might be transferable to the clinic whereby co-administration of a CYP2D6 inhibitor with initial haloperidol exposure may reduce acute toxicity. In rats, chronic haloperidol exposure increases vacuous chewing movements, a model of the chronic side effect tardive dyskinesia (Miksys et al, 2017). In contrast to acute haloperidol side effects, brain CYP2D appears to be neuroprotective for tardive dyskinesia-like side effects caused by chronic haloperidol; inhibiting brain CYP2D during chronic haloperidol administration increased vacuous chewing movements (Miksys et al, 2017). This suggests that haloperidol itself is responsible for this chronic side-effect, or a metabolite made by an alternative enzyme, and that having more brain CYP2D is protective during long term haloperidol exposure (Figure 3). The difference in underlying neurochemistry between haloperidol-induced acute (catalepsy, likely mediated at least in part by a CYP2D-mediated metabolite) and chronic (VCMs, likely mediated by the parent drug haloperidol, or a metabolite via a non-CYP2D-mediated pathway) neurotoxicity may be one reason behind the opposing role of brain CYP2D metabolism in acute (a risk factor) and chronic (a protection factor) side effects.
Figure 3: Inhibiting in vivo rat brain CYP2D activity differentially alters extrapyramidal side-effects from acute and chronic haloperidol. Rat brain CYP2D was inhibited by a single ICV pretreatment of propranolol (PL, CYP2D mechanism-based inhibitor given 24 h ahead of haloperidol; illustrated in a), or chronically inhibited with ICV propranolol 3 times per week (with chronic haloperidol over 16 weeks; illustrated in e). Inhibiting brain CYP2D with ICV propranolol (PL) reduced catalepsy from a single haloperidol injection, likely mediated by reduced central metabolite production. Catalepsy time was shorter (b), indicating decreased neurotoxicity, and correlated positively with brain (c) but not liver (d) CYP2D activity. Brain CYP2D inhibition during chronic haloperidol exposure increased vacuous chewing movements (VCMs), likely mediated by increased brain parent drug, haloperidol. VCMs over the last 4 weeks were higher (f), indicating increased neurotoxicity, and correlated negatively with brain (g) but not liver (h) CYP2D activity. The full study is described in Miksys et al. (2017). *p < 0.05 vs control.

3.3. Brain CYP-mediated metabolism of endogenous compounds and neurotransmitters

An initial indication that brain CYP-mediated metabolism of endogenous compounds may impact physiological function was derived from the association between CYP2D6 genotype and personality (Penas-Lledo and Llerena, 2014). For example, compared to CYP2D6extensive metabolizers, CYP2D6 poor metabolizers have lower brain perfusion rates (an index of cerebral activity) in the thalamus and hippocampus, indicative of lower metabolic activity, which associates with displayed anxious traits (Kirchheiner et al, 2011). CYP2D6genotype was also associated with
cortical brain activation in multiple cognitive tests (working memory and emotional face matching), suggesting a role for brain CYP2D6 in brain information processing (Stingl et al., 2012).

The relationship between CYPs and endogenous neurotransmitter systems is complex (Brtko and Dvorak, 2011; Dvorak and Pavek, 2010; Waxman and O'Connor, 2006; Wojcikowski et al., 2007). Brain CYP2D6 can biosynthesize the monoaminergic neurotransmitters dopamine and serotonin in vitro; this may represent an additional pathway contributing to total synthesis of these brain neurotransmitters (Hiroi et al., 1998b; Yu et al., 2003). Formation of dopamine via tyramine hydroxylation in rat brain microsomes was inhibited by the CYP2D inhibitor quinine and anti-CYP2D4 antibodies (rat CYP2D4 is a potential homologue of human CYP2D6) (Bromek et al., 2010). Using cDNA-expressed rat CYPs and human CYP2D6, as well as rat brain microsomes, CYP2Ds were found to be the most efficient CYPs in forming serotonin from 5-methyltryptamine; this formation could be inhibited by the CYP2D inhibitors quinine and fluoxetine (Haduch et al., 2013). Compared to cDNA-expressed wild-type CYP2D6 (in recombinant E. coli), allelic variants of CYP2D6 (CYP2D6*10 and *12, associated with deficient activity) exhibited decreased 6β-hydroxylation of progesterone and decreased formation of dopamine from tyramine (Niwa et al., 2004).

Rat brain CYP2D formation of dopamine and serotonin in vivo has been assessed using microdialysis (Bromek et al., 2011; Haduch et al., 2015). Following enhancement of the potential role of brain CYP2D via ablation of the classic pathways of dopamine and serotonin production (e.g. inhibition of dopamine formation from tyrosine with α-methyl-p-tyrosine, and inhibition of serotonin formation from tryptophan with the serotonergic neurotoxin 5,7-dihydroxytryptamine) there was some evidence that brain CYP2D could create dopamine and serotonin from exogenously administered dopamine and serotonin precursors (e.g. tyramine or 5-methyltryptamine and melatonin) (Figure 4) (Bromek et al., 2011; Haduch et al., 2015; Haduch et al., 2016). Humanized Tg-2D6 mice (as mentioned previously, with human CYP2D6 protein expressed in the liver and brain) have higher brain basal serotonin levels relative to wild-type mice (Cheng et al., 2013). So, while there is currently no direct evidence for a role of brain CYP2D metabolism in endogenous neurotransmitter and neurosteroid production or removal, studies of human genetic associations, exogenous precursor metabolism, and humanized mice provide
motivation to continue to examine this potential role for brain CYP-mediated metabolism in neurochemical homeostasis.

**Figure 4:** Brain CYP2D may contribute to in vivo central metabolism of exogenous tyramine to dopamine. When the primary dopamine formation pathway from tyrosine, and dopamine catabolic pathways, were inhibited (a), the alternate pathway of brain CYP2D-mediated dopamine formation from tyramine was isolated (b). Exogenous tyramine was given via microdialysis probe directly into the striatum with or without administration of a striatal injection of the CYP2D inhibitor quinine (Q; illustrated in b). Compared to controls (grey), brain CYP2D inhibition had no effect on dopamine levels (grey with red border; c: % of basal level, d: AUC$_{0-240}$ min). However, inhibiting brain CYP2D in the presence of exogenous tyramine, which results in increased brain dopamine levels (green; c, d), attenuated the increase in dopamine formation (green with red border; c, d). The full experimental results can be found in Bromek et al. (2011). *p < 0.05, **p < 0.001 vs control; #p < 0.001 vs exogenous tyramine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In vitro, CYP2C19 metabolizes endogenous (e.g. estradiol, progesterone, and testosterone) and synthetic (e.g. selective serotonin reuptake inhibitors sertraline and citalopram) psychoactive compounds (Ingelman-Sundberg et al., 2007) and is expressed in fetal, but not adult, human brain (Persson et al., 2014). Humans homozygous for the loss of function CYP2C19*2 allele, who do not express CYP2C19 enzyme, have a lower incidence of depressive symptoms, relative to CYP2C19 extensive metabolizers (CYP2C19*1/*1) (Sim et al., 2010). The hippocampus is
critical in inhibiting stress responses and reduced hippocampal volume has been found in mood disorders like major depressive disorder (Femenia et al., 2012). Transgenic male mice (CYP2C19Tg-Hem) that are hemizygous for human CYP2C19 display greater anxiety and impairment of hippocampal serotonin turnover (indicated by 5-HIAA (metabolite)/serotonin) and brain-derived neurotrophic factor homeostasis as well as reduced hippocampal volume and adult neurogenesis (Jukic et al., 2016; Persson et al., 2014). Although CYP2C19 is expressed in adult human brain at negligible levels, genetically variable CYP2C19 enzyme function may alter normal hippocampal activity during neurodevelopment (Toselli et al., 2015). While the mechanism for the impact of CYP2C19 on hippocampal development is unknown, metabolism of endogenous compounds involved in overall brain development (e.g. fatty acids, sex hormones) may contribute (Persson et al., 2014).

CYP3A4 can also metabolize neuroactive steroids (e.g. testosterone and estradiol) thereby having the potential to influence mood, behaviour, memory, and cognition (Lee et al., 2003; Wang et al., 1997). The incidence of mood disorders and cognitive deficits is increased in epilepsy patients compared to healthy controls, as well as epilepsy patients treated with anti-epileptics (Beyenburg et al., 2005; Isojarvi et al., 2005; Verrotti et al., 2011). Human CYP3A4 is higher in post mortem brains of individuals exposed to anti-epileptic drugs (e.g. carbamazepine and phenytoin); likewise, mouse CYP3A11 is induced by anti-epileptic drugs in a hippocampal cell line (HN25.1) (Killer et al., 2009). Administration of anti-epileptic drug phenytoin to mice induced CYP3A and androgen receptor expression in the hippocampus (Meyer et al., 2006). Compared to controls, phenytoin treated mice had lower brain testosterone levels, suggesting that induced brain CYP3A increased testosterone metabolism, thereby yielding a compensatory increase in androgen receptor expression (Meyer et al., 2006). Similarly, rat brain CYP2E1 was induced by phenytoin, and following acutely-induced status epilepticus by kainic acid (Boussadie et al., 2014).

Other CYPs expressed in the brain may also play a role in modulating CNS active compounds. CYP1A1 and CYP1A2 metabolize numerous endogenous compounds (e.g. arachidonic acid, other eicosanoids, estrogens, bilirubin, and melatonin) (Rifkind, 2006). In addition to exogenous opioid analgesia previously discussed, neuronal CYP (e.g. CYP2C, CYP2J
and CYP4X1) epoxygenation of AA to EET plays a role in endogenous opioid-mediated stress-induced analgesia (Hough et al, 2014b). Compared to control mice, neuronal CYP-deficient (null) mice exhibited less stress-induced analgesia (as measured by the restraint model of stress-induced analgesia), similar to the decrease in stress-induced analgesia after pretreatment with the opioid antagonist naltrexone (Hough et al, 2014b). EETs exhibit anti-inflammatory properties and are involved in protection against stroke, cerebral ischaemia and pain (Iliff et al, 2010). Exogenous glutamate increased rat brain CYP2J2 mRNA levels and the sum of EET and dihydroxyeicosatrienoic acids, indicative of increased CYP2J2 EET production (Liu et al, 2017); altered CYP-metabolic production of EETs in vivo may be an important drug-target for treating various disease states. Alternatively, CYP4A and CYP4F can produce hydroxyeicosatetraenoic acids (HETEs, e.g. 20-HETE) by hydroxylating AA; 20-HETE elicits a wide range of organ-specific effects on the human vascular system (e.g. maintaining preglomerular vascular tone in the kidney) (Elshenawy et al, 2017). Brain metabolism of AA to 20-HETE occurs mainly through CYP2U1, which is abundant in human cerebellum (Chuang et al, 2004). The human brain does not store glycogen as a reservoir of energy, but instead requires a continuous supply of glucose and oxygen; regulation of brain vascular tone by 20-HETE (e.g. through brain CYP2U1 production from AA) may play a role in sustaining normal brain function (Elshenawy et al, 2017; Pratt et al, 2004).

4. Expression and activity of brain CYPs

CYPs are expressed, and active, in the brains of a wide-range of species (Meyer et al, 2007). The cellular distribution and expression levels of brain CYPs can vary across brain regions and species; we have outlined some of these differences where important, however more detailed species comparisons can be found in prior reviews (Toselli et al, 2016). While hepatic CYPs are primarily expressed in the endoplasmic reticulum, brain CYPs are found on mitochondrial membranes, the plasma membrane, and other cell compartment membranes (Walther et al, 1986). CYP1B1 is expressed at the human BBB, where it may act in conjunction with transport proteins by regulating xenobiotic permeation (Dauchy et al, 2008; Decleves et al, 2011; Dutheil et al, 2009). CYP2B expression in human brain is highest in the dentate gyrus of the hippocampus and in astrocytes adjacent to cerebral blood vessels; here it may play a similar role to CYP1B1 in neuroprotection at the BBB by acting with transporters (Miksys et al, 2003). CYP2B expression in monkey brain is
highest in pyramidal cells of the cortex, Purkinje cells in the cerebellum, and neurons in the substantia nigra (Lee et al., 2006b), while expression in rats and mice is highest in neurons of the cerebellum, hippocampus, and thalamus (Volk et al., 1991), suggesting some species differences in CYP distribution.

CYP2D expression in human and monkey brain is primarily located in pyramidal neurons, with high expression in the cortex, cerebellum, and hippocampus (Mann et al., 2008; Miksys et al., 2002). In rat and mouse brain, CYP2D is widely expressed in neuronal and glial populations heterogeneously across brain regions, including regions unprotected by the BBB, such as the choroid plexus (Miksys et al., 2000a; Miksys et al., 2005; Norris et al., 1996). CYP2E1 shares a relatively similar expression profile in rat, monkey, and human brain; for example, CYP2E1 protein is expressed in pyramidal neurons of the frontal cortex, cerebellum, and hippocampus (Garcia-Suastegui et al., 2017; Hansson et al., 1990; Joshi and Tyndale, 2006b; Tindberg and Ingelman-Sundberg, 1996; Upadhya et al., 2000).

Transcripts for CYP1A2 and the orphan CYP2S1 and CYP4X1 have been detected in rat hippocampus, cortex, and cerebellum (Stamou et al., 2014). Mouse neurons express CYP4A10 and CYP4A12a, which metabolize AA and may function to protect the brain from ischemic injury (e.g. oxygen-glucose deprivation) (Zhang et al., 2017).

5. Regulation of brain CYPs

A unique feature of brain CYPs is their sensitivity to xenobiotic inducers, which may differ from induction of liver CYPs. Regulation of brain CYPs is region-specific, depending on CYP isoform and inducer (Miksys and Tyndale, 2004). For example, smokers and alcoholics have higher levels of brain CYP2B6 in the hippocampus, caudate nucleus, putamen, and cerebellum, with greater increased expression in individuals classified as both smokers and alcoholics (Miksys et al., 2003). Repeated nicotine administration (7 days) increases rat brain CYP2B expression and mRNA, with the largest increases occurring in brain stem, frontal cortex, striatum, and olfactory tubercle (Miksys et al., 2000a). Nicotine pretreatment did not alter rat liver CYP2B expression or mRNA levels (Khokhar et al., 2010). The increase in rat brain CYP2B protein and mRNA levels after nicotine pretreatment was not affected by inhibition of nicotinic-acetylcholine receptor (nAChR)
with chlorisondamine (nAChR irreversible inhibitor), indicating that the mechanism of nicotine induction of brain CYP2B may be through increased transcription, independent of nAChR activation (Khokhar et al, 2010). Monkey brain CYP2B6 is induced by nicotine (alone and in combination with ethanol) with no apparent increase in brain CYP2B6 mRNA levels, suggesting a species-specific difference in regulation (Ferguson et al, 2013).

While liver CYP2D activity in humans is primarily determined by genetics, brain CYP2D levels can be altered by both genetics and xenobiotic inducers. Smokers have higher CYP2D6 levels, as assessed in post-mortem brain, while liver CYP2D levels do not differ between smokers and non-smokers (Mann et al, 2008; Miksys et al, 2002; Miksys and Tyndale, 2004). Nicotine pretreatment (7 days) increases rat brain CYP2D expression, with the largest increases in the cerebellum, hippocampus, and striatum (Yue et al, 2008). Nicotine and ethanol dose-dependently increase monkey brain CYP2D expression (Mann et al, 2008; Miller et al, 2014). Neither rat nor monkey brain CYP2D increases were associated with changes in brain CYP2D mRNA levels, and neither hepatic enzyme activity nor mRNA levels were changed, suggesting a brain-specific effect on CYP2D by non-transcriptional regulation (Mann et al, 2008; Miller et al, 2014; Yue et al, 2008). The mechanism of brain CYP2D induction is currently unknown, but may involve increased protein stability or decreased protein degradation; chronic (14 day) nicotine administration downregulates ubiquitination enzymes in rat hypothalamus which suggests one possible mechanism (Kane et al, 2004). Brain CYP2E1 may be differently regulated by nicotine and ethanol; ethanol treatment induces rat brain CYP2E1 levels and mRNA, suggesting a transcriptional mechanism of regulation, while induction of CYP2E1 by nicotine is not accompanied by an increase in mRNA and therefore may involve translational efficiency or protein stability (Howard et al, 2003; Joshi and Tyndale, 2006a; Zhong et al, 2012). Mitogen-activated protein kinase signaling may play a role in ethanol induction of brain CYP2E1; inhibitors of the p38 and ERK1/2 signaling pathways attenuated the induction of rat brain CYP2E1 mRNA and protein levels by ethanol (Na et al, 2017). Nicotine and ethanol alone, and in combination, increased monkey brain CYP2E1 levels with no change in mRNA, again suggesting a non-transcriptional mechanism of regulation (Ferguson et al, 2013). In the case of ethanol, there is a potential species or dosing paradigm difference between rat and monkey brain CYP2E mechanism of regulation by xenobiotic inducers (Ferguson et al, 2013). These findings are consistent with the
higher brain CYP2E1 levels observed in alcoholics and smokers compared to non-alcoholics and non-smokers (Ferguson et al, 2013; Howard et al, 2003; Joshi and Tyndale, 2006b; Warner and Gustafsson, 1994).

Many known xenobiotic inhibitors and inducers of liver CYP expression have effects on brain CYP expression as well. Prolonged exposure to antidepressants (imipramine and mirtazapine) and neuroleptics (thioridazine and risperidone) decreases CYP2D activity and expression in rat brain and liver (Haduch et al, 2011; Haduch et al, 2004). Similarly, the neuroleptic clozapine and solvent toluene induce rat CYP2D expression in brain and liver (Hedlund et al, 1996). Phenobarbital increases the expression and activity of rat brain CYP2B1, CYP2B2, and CYP3A1, and has been shown to increase monkey brain CYP2B6; phenobarbital also increases liver CYP2B expression in both species (Lee et al, 2006c; Schilter et al, 2000). Phenobarbital increases monkey brain CYP2E1 expression in the cerebellum and putamen, with no alteration of liver CYP2E1 protein levels or in vivo chlorzoxazone (CYP2E1 probe drug) disposition (Lee et al, 2006a). In contrast, rat liver CYP2E1 mRNA is increased with phenobarbital exposure, again suggesting species-dependent (or dose-dependent) effect of xenobiotics on CYP expression (Caron et al, 2005b).

Brain CYP expression is further regulated by endogenous factors (e.g. developmental or hormonal). Liver CYP2D6 levels in humans increase rapidly after birth to adult levels and remain constant with age (Treluyer et al, 1991), however CYP2D6 enzyme levels assessed in frontal cortex increase with age from fetal stages to 80 years of age, which may occur in other brain regions as well (Mann et al, 2012). Human sex-based differences in liver CYP expression vary between CYP isoforms; sex hormones may play a role in regulating brain CYP expression (Aichhorn et al, 2005; Hildebrandt et al, 2003; Waxman and Holloway, 2009). Exogenous estrogen and testosterone increases, while progesterone decreases, brain CYP2D expression and mRNA levels in female rats (Baum and Strobel, 1997; Bergh and Strobel, 1996). In contrast to this, testosterone decreases CYP2D6 activity in human cell lines (SH-SY5Y and U251) and orchiectomized growth hormone receptor knockout (GHR-KO) male mice and rats (Li et al, 2015a). In liver, the expression of some CYPs is regulated by xenobiotic receptors (e.g. aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor (PPAR), and retinoid X
receptor (RXR)) (Honkakoski and Negishi, 2000). In brain, the mRNA of numerous receptor subtypes has been detected (e.g. PPAR subtypes β and δ, RXRβ and AhR) (Nishimura et al., 2004; Petersen et al., 2000). Their expression varies among brain regions, is cell-specific, and is genetically variable (Jeninga et al., 2009; Koyano et al., 2005; Lamba et al., 2005; Lamba et al., 2004a; Lamba et al., 2004b; Moreno et al., 2004; Petersen et al., 2000). Expression of nuclear receptors in brain can be modulated by other receptors such as the estrogen receptor, and there can be cross-talk among receptors (Tetel, 2009; Xiao et al., 2010). The role of nuclear receptors in regulating brain CYP expression, independent of liver CYPs, is currently poorly understood.

6. Conclusions
The functional role of CYP enzymes in the brain is a new, exciting and quickly evolving area of research. CYPs are present and active in the brain, with heterogeneous patterns of expression, activity, and sensitivity to modulation across cell types, regions, and species. Despite total brain CYP expression being a fraction of hepatic, the expanding literature on brain CYP-mediated metabolism has shown that brain CYPs can impact acute (e.g. codeine analgesia (McMillan and Tyndale, 2015; Zhou et al., 2013)) and chronic (e.g. nicotine dependence Garcia, et al., 2015)) response to drugs, susceptibility to damage by neurotoxins, and are associated with altered personality and behaviour, and risk of neurological disease; they may also play a role in neurotransmitter levels. The multifaceted impact of CYP-mediated brain metabolism is delineated in the review figures; for any single CYP enzyme active in the brain it may be involved in drug activation (e.g. Figure 2), protection against neurotoxicity (e.g. Figure 3), and/or neurotransmitter metabolism (e.g. Figure 4). Understanding the functional role for brain CYP-mediated metabolism in vivo has been aided by the development of animal models that allow modulation of brain-specific CYP activity without affecting CYP activity in the liver. This includes (but is not limited to) direct ICV injection of mechanism-based inhibitors (e.g. C8-xanthate against rat brain CYP2B (Miksys and Tyndale, 2009) and propranolol against rat brain CYP2D (Zhou et al., 2013)), peripherally administered inducers of brain CYP activity (e.g. chronic sub-cutaneous nicotine induction of rat brain CYP2D (Yue et al., 2008)), and brain-specific CYP knockdown models (e.g. brain-Cpr-null mice (Conroy et al., 2010)). The mechanisms behind the sensitivity of brain CYPs to xenobiotic inducers are still poorly understood. The use of in vivo brain microdialysis as a technique for assessing brain metabolism in real-time should prove to be a useful additional tool,
for example to assess rat brain nicotine levels after brain CYP2B inhibition (Garcia et al, 2015) and induction (Garcia et al, 2016). Animal imaging studies, as well as human studies which incorporate human CYP genetic variants, may further elucidate a role for brain CYPs. For example, neuroimaging studies have begun to explore the link between CYP2D6 polymorphisms and basic brain function, via tests of working memory and facial expression recognition (Stingl et al, 2013). Understanding brain CYP function may eventually produce novel strategies for the development of therapeutics that incorporate not only the consequences of metabolism by brain CYPs, but also the therapeutic targeting of brain CYP-mediated metabolism.

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