CYP2D is Functional in the Brain and Alters Haloperidol-Induced Side Effects

by

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Department of Pharmacology and Toxicology
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Abstract

The human cytochrome P450 2D6 (CYP2D6) enzyme metabolizes numerous neurotoxins and centrally-acting drugs, including first generation antipsychotics such as haloperidol. These antipsychotics are used to treat schizophrenia and can cause adverse events in patients following acute and chronic intake. CYP2D6 expression in the liver is essentially uninducible and is regulated by genetics, whereas CYP2D6 expression in the brain is dependent on many factors, including *CYP2D6* genetic variation, brain-specific induction of CYP2D6, and drug-drug interactions. Variation in human CYP2D6 activity in the brain may alter local drug and metabolite levels sufficiently to change drug response, including therapeutic efficacy and adverse events. This thesis investigated the impact of brain-specific manipulation of CYP2D on haloperidol-induced responses. CYP2D in rat brain (but not in rat liver) was inhibited by 24-hour pre-treatment with intracerebroventricular propranolol and induced by 7-day subcutaneous nicotine treatment. We found that acute haloperidol-induced catalepsy was decreased after inhibiting, and increased after inducing, CYP2D selectively in rat brain. In contrast, chronic haloperidol-induced vacuous chewing movements were increased after inhibiting, and decreased after inducing, CYP2D selectively in rat brain. Further, we investigated human CYP2D6 drug metabolism *in vivo* using CYP2D6-transgenic mice (TG) which express human CYP2D6 and

mouse CYP2D, and in wildtype mice (WT). Mouse CYP2D and human CYP2D6 in TG liver, and mouse CYP2D in WT liver, were inhibited by 24-hour pre-treatment with intraperitoneal propranolol. In contrast, a 24-hour pre-treatment with intracerebroventricular propranolol irreversibly inhibited human CYP2D6 in TG brain, but not mouse CYP2D in TG or WT brain; there was no inhibition of hepatic CYP2D in TG or WT. Brain-specific inhibition of human CYP2D6 in TG brain reduced acute haloperidol-induced catalepsy, without affecting plasma haloperidol levels. This thesis demonstrated that CYP2D, including human CYP2D6, in the brain plays a role in *in vivo* haloperidol-induced responses. We have developed a new tool that enables the investigation of human CYP2D6 in brain on *in vivo* drug-response. In conclusion, we demonstrated that human CYP2D6 in the brain is functional and sufficiently able to alter drug-induced behaviour, suggesting a role in altering therapeutic efficacy and adverse events in humans.

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Declaration

I, Cole Tolledo, hereby declare that I composed this thesis, that the work contained herein is my own, except where stated otherwise, and that this work has not been submitted for any previous degree. Much of the text included here is taken directly from our published papers (see publication list).

Cole Tolledo's Contributions to the Thesis

Study 1. "**CYP2D enzymatic metabolism in the rat brain alters acute and chronic haloperidol side-effects by different mechanisms"**. This work has been published (Miksys et al. Prog. Neuropsychopharmacol. Biol. Psychiatry. 2017). Cole Tolledo designed the *ex vivo* brain and liver incubation experiments, performed these experiments, and analyzed the data. Cole Tolledo critically reviewed, edited, and approved the final version of the publication.

Study 2. "Propranolol is a mechanism-based inhibitor of CYP2D and CYP2D6 in humanized CYP2D6-transgenic mice: effects on activity and drug response". This work has been published (Tolledo et al. Br. J. Pharmacol. 2020). Cole Tolledo, along with other authors, designed the study. Cole Tolledo optimized and performed the *in vitro* propranolol inhibition experiments, and optimized and performed the *ex vivo* liver incubation experiments. Cole Tolledo, with support, collected blood samples for *in vivo* plasma dextromethorphan and dextrorphan levels after an *in vivo* 24-hour pre-treatment with intraperitoneal propranolol, performed the acute haloperidol-induced catalepsy experiments, and collected blood samples for *in vivo* plasma haloperidol levels after an *in vivo* 24-hour pre-treatment with intraperitoneal propranolol. Cole Tolledo statistically analyzed the data and wrote the manuscript.

Study 3. "Human CYP2D6 is functional in brain *in vivo*: evidence from humanized CYP2D6-transgenic mice". This work has been published (Tolledo et al. Mol. Neurobiol. 2020). Cole Tolledo, along with other authors, designed the study. Cole Tolledo optimized the *ex vivo* brain and liver incubation experiments, and performed the *ex vivo* liver incubation experiments. Cole Tolledo, with support, performed the *ex vivo* brain incubation experiments, collected and processed brain tissue for *in vivo* brain dextromethorphan and dextrorphan levels, and collected and processed blood samples for *in vivo* plasma dextromethorphan and dextrorphan levels after an *in vivo* 24-hour pre-treatment with intracerebroventricular propranolol. Cole Tolledo, with support, performed the acute haloperidol-induced catalepsy experiments and collected blood samples for *in vivo* plasma haloperidol levels after an *in vivo* 24-hour pre-treatment with intracerebroventricular propranolol. Cole Tolledo, and others, performed the cannulation surgeries. Cole Tolledo analyzed the data and wrote the manuscript.

Appendix A. "**Human CYP2D6 in the brain is protective against harmine-induced neurotoxicity: evidence from humanized CYP2D6 transgenic mice."** This work has been

submitted. Cole Tolledo, along with other authors, designed the study. Cole Tolledo optimized the *ex vivo* brain and liver incubation experiments, and performed the *ex vivo* liver incubation experiments. Cole Tolledo contributed to the *ex vivo* brain incubation experiments. Cole Tolledo, and others, performed the cannulation surgeries. Cole Tolledo analyzed the *ex vivo* brain and liver incubation data. Cole Tolledo critically reviewed, edited, and approved the final version of the publication.

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Research Articles in Thesis

Study 1: Miksys, S., Wadji, F.B., **Tolledo, E.C.,** Remington, G., Nobrega, J.N., Tyndale, R.F., 2017. Rat brain CYP2D enzymatic metabolism alters acute and chronic haloperidol side-effects by different mechanisms. Progress in Neuro-Psychopharmacology and Biological Psychiatry 78, 140-148.

Study 2: **Tolledo, E.C.,** Miksys, S., Gonzalez, F.J., Tyndale, R.F., 2020. Propranolol is a mechanism-based inhibitor of CYP2D and CYP2D6 in humanized CYP2D6-transgenic mice: effects on activity and drug response. British Journal of Pharmacology 177(3), 701-712.

Study 3: **Tolledo, C.,** Stocco, M.R., Miksys, S., Gonzalez, F.J., Tyndale, R.F., 2020. Human CYP2D6 Is Functional in Brain In Vivo: Evidence from Humanized CYP2D6 Transgenic Mice. Molecular Neurobiology 57(6), 2509-2520.

Additional Manuscripts from Doctoral Work Attached in Appendix

Stocco, M.R., **Tolledo, C.,** Wadji, F.B., Gonzalez, F.J., Miksys, S., Tyndale, R.F., 2020. Human CYP2D6 in the brain is protective against harmine-induced neurotoxicity: evidence from humanized CYP2D6 transgenic mice. *Submitted.*

Submitted Abstracts for Oral and Poster Competitions

Tolledo, E.C., Tyndale, R.F. Oral: Propranolol is a mechanism-based inhibitor of CYP2D and CYP2D6 in humanized transgenic mice: effects on activity and drug response. June 2019 – University of Toronto Department of Pharmacology Visions in Pharmacology (VIP); Toronto, ON, Canada.

Tolledo E.C., Stocco, M.R., Miksys, S., Gonzalez, F.J., Wadji, F.B., Zhao, B., Tyndale, R.F. Poster: A mouse model for investigating human brain CYP2D6. July 2018 – 22nd North American International Society for the Study of Xenobiotics Meeting, Montreal, QC, Canada

Tolledo E.C., Stocco, M.R., Miksys, S., Gonzalez, F.J., Wadji, F.B., Zhao, B., Tyndale, R.F. Poster: A mouse model for investigating human brain CYP2D6. June 2018 – University of Toronto Department of Pharmacology Visions in Pharmacology (VIP); Toronto, ON, Canada.

Tolledo E.C., Miksys, S., Wadji, F.B., Gonzalez, F.J., Novalen, M., Zhao, B., Tyndale, R.F. Poster: CYP2D activity in liver and brain of C57BL/6 and CYP2D6 transgenic mice. October 2016 – 21st International Symposium on Microsomes and Drug Oxidation, Davis, CA, USA

Tolledo E.C., Miksys, S., Wadji, F.B., Novalen, M., Zhao, B., Tyndale, R.F. Poster: Characterization of CYP2D activity in brain and liver of C57BL/6 mice. June 2016 – University of Toronto Department of Pharmacology Visions in Pharmacology (VIP); Toronto, ON, Canada.

Tolledo E.C., Miksys, S., Salahpour, A., Tyndale, R.F. Poster: Cytochrome P450 2D6 enzyme in the brain: neuroprotection against Parkinson-causing neurotoxins. June 2015 – University of Toronto Department of Pharmacology Visions in Pharmacology (VIP); Toronto, ON, Canada.

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List of Abbreviations

AUC	Area under the curve
CL_{int}	Intrinsic clearance
CYP	Cytochrome P450
CYP2D6	Cytochrome P450 2D6
EM	Extensive metabolizer
HPLC	High performance liquid chromatography
HPP^{+}	4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-pyridinium
IC_{50}	Half-maximal inhibitor concentration
icv	Intracerebroventricular
im	Intramuscular
IM	Intermediate metabolizer
ip	Intraperitoneal
kDa	kilodalton
K_i	Inhibitor binding affinity
$K_{inactivation}$	Maximal inactivation rate
K_I	Inactivator binding affinity
K_m	Substrate binding affinity
K_{obs}	Initial rate constant of inactivation of dextrorphan formation by each propranolol concentration
LCMS	Liquid chromatography-mass spectrometry
MBI	Mechanism-based inhibitors
MPP^{+}	(1-methyl-4-phenylpyridinium)
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PM	Poor metabolizer
$RHPP^{+}$	4-(4-chlorophenyl)-1-[4-(fluorophenyl)-4-hydroxybutyl]-pyridinium
sc	Subcutaneous
TG	CYP2D6-Transgenic mice
UM	Ultra-rapid metabolizer
V_{max}	Maximal substrate turnover rate
WT	C57BL/6 Wildtype mice

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Stocco, M.R., **Tolledo, C.,** Wadji, F.B., Gonzalez, F.J., Miksys, S., Tyndale, R.F., 2020. Human CYP2D6 in the brain is protective against harmine-induced neurotoxicity: evidence from humanized CYP2D6 transgenic mice. *Submitted.*

1 Introduction

Statement of Research Problem

Cytochrome P450 2D6 (CYP2D6) is expressed in both the liver and the brain (Miksys and Tyndale, 2013). CYP2D6 metabolizes a large number of substrates including centrally-acting drugs such as antipsychotics, antidepressants, and opioids (Zanger and Schwab, 2013). In addition, CYP2D6 also metabolizes endogenous ligands such as trace amines (Hiroi et al., 1998b; Yu et al., 2003a) and neurosteroids (Hiroi et al., 2001). CYP2D6 also inactivates neurotoxins such as Parkinsonian toxins (Coleman et al., 1996; Modi et al., 1997), β-carbolines (Herraiz et al., 2006; Yu et al., 2003b), and tetrahydroisoquinolines (Suzuki et al., 1992). Hepatic CYP2D6 is considered uninducible, and hepatic CYP2D6 levels and activity are solely regulated by genetics (Edwards et al., 2003; Madan et al., 2003). The *CYP2D6* gene is highly genetically polymorphic and results in activity that ranges from very high activity in *CYP2D6* ultra-rapid metabolizers (UMs) to very little to no activity in *CYP2D6* poor metabolizers (PMs) (Gaedigk, 2013). Therefore, the metabolism of a number of centrally acting drugs that are CYP2D6 substrates, such as antipsychotics, also varies greatly between individuals. *CYP2D6* genotype greatly contributes to interindividual variability in pharmacokinetics, and it is well characterized that plasma levels of psychotropic drugs are greatly influenced by *CYP2D6* genotype (Bertilsson et al., 2002). However, *CYP2D6* genotype is not the only contributor to therapeutic effect, or adverse drug reaction incidence and severity.

Adverse drug reactions such as parkinsonism and tardive dyskinesia from first-generation antipsychotics are not uncommon (Leucht et al., 2012). First-generation antipsychotics such as haloperidol are CYP2D6 substrates that are used to treat positive symptoms of schizophrenia (Marder and Cannon, 2019), a serious and debilitating psychiatric disorder. *CYP2D6* PMs who take first generation antipsychotics (Brockmöller et al., 2002; Chou et al., 2000; de Leon et al., 2005; Meyer et al., 1990; Schillevoort et al., 2002; Spina et al., 1992; Tamminga et al., 2003) have higher occurrences of antipsychotic-induced adverse reactions compared to *CYP2D6* extensive metabolizers (EMs), individuals with *CYP2D6* genotype that results in CYP2D6 activity that is faster than *CYP2D6* intermediate metabolizers (IMs) and PMs, but slower than *CYP2D6* UMs. Since CYP2D6 can metabolize antipsychotics such as haloperidol, it is possible that CYP2D6 may influence the occurrence of adverse events.

While the liver is primarily responsible for the metabolism of the majority of drugs in the body, plasma drug and metabolite levels do not always correlate with observed therapeutic response, especially with centrally-acting drugs (Michels and Marzuk, 1993). Brain-specific CYP2D6 expression, combined with the passage of active drug and/or metabolite across the naturally permeable blood-brain barrier, may allow CYP2D6 in the brain to metabolize drugs that target the brain, thus altering brain levels of both the parent drug and metabolite. In contrast to CYP2D6 levels in the liver, CYP2D6 levels in the brain are influenced not just by genetics, but also by age (Mann et al., 2012) and environmental exposures (Mann et al., 2008; Miksys et al., 2002; Miksys and Tyndale, 2004). For example, chronic exposure to nicotine induces rat and monkey CYP2Ds in the brain without affecting the liver (Mann et al., 2008; Miller et al., 2014; Yue et al., 2008), similar to findings that human smokers have higher human CYP2D6 levels in the brain (Mann et al., 2008; Miksys and Tyndale, 2004) but similar CYP2D6 levels in the liver compared to non-smokers (Bock et al., 1994; Funck-Brentano et al., 2005; Steiner et al., 1985). Thus, the interaction between genetics, age, environmental exposures, and exposure to substrates that can inhibit or induce human CYP2D6, may result in variation in CYP2D6 activities in the brain between people, without altering CYP2D6 in the liver. In turn, this variation in CYP2D6 in the human brain may result in altered brain drug and metabolite levels, which may not be reflected in altered peripheral drug and metabolite levels, possibly mediating therapeutic and adverse drug responses to centrally-acting drugs metabolized by CYP2D6.

The impact of variable metabolism of opioids by CYP2D in the brain has been examined in rats, where the effects of inhibition via propranolol and induction via nicotine of CYP2D activity in the brain, without changing CYP2D activity in the liver, were assessed (McMillan et al., 2019; McMillan and Tyndale, 2015, 2017; Zhou et al., 2013). The inhibition of CYP2D in rat brain changes brain, but not plasma, drug levels, and accompanying opioid-induced analgesia from the CYP2D substrates codeine (McMillan and Tyndale, 2015; Zhou et al., 2013), and oxycodone (McMillan et al., 2019). Thus, it is possible that occurrence of adverse events from first generation antipsychotics could be affected by brain-specific metabolism by CYP2D6 in the brain.

The ultimate goal of this work is to gain a better understanding of the general role of CYP2D in brain, and the specific role of human CYP2D6 in the brain. Using manipulation of CYP2D activity in rodent brain, we can test the impact of local CYP2D-mediated metabolism of

drugs on their corresponding behaviours, providing evidence that CYP2D in the brain plays a role in resulting therapeutic effects and the occurrence of adverse-events. The work in this thesis focused on extending findings in models of variable (increased and decreased) CYP2D activity in both rat and mouse brain. The rat paradigm of manipulating CYP2D in the brain, but not CYP2D in the liver, via intracerebroventricular propranolol inhibition and via nicotine induction, was applied to haloperidol metabolism within the brain and resulting behaviours. This study looked at both acute and chronic haloperidol treatments and measured behavioural responses, catalepsy and vacuous chewing movements, respectively, which model adverse events experienced by patients taking first generation antipsychotics. This work was then extended in CYP2D6-transgenic mice (Cheng et al., 2013), which express human CYP2D6 in addition to mouse CYP2D isozymes, and in C57BL/6J wild-type mice. To adapt this paradigm of selectively and irreversibly inhibiting CYP2D to mouse brain, *in vivo* CYP2D inhibition was first characterized in the liver and subsequently characterized in the brain. The studies in this thesis sought to provide evidence for a role for CYP2D, and specifically human CYP2D6, *in vivo* on brain drug metabolism and resulting behaviour, as well as provide tools that may allow future work to directly test the role that CYP2D6 in the brain plays with regards to drug response, neurotoxicity, and endogenous neurotransmitter metabolism.

Purpose of the Study and Objectives

Study 1. "**CYP2D enzymatic metabolism in the rat brain alters acute and chronic haloperidol side-effects by different mechanisms"**. Antipsychotics exert their therapeutic effects by blocking striatal dopamine D2 receptors, resulting in extrapyramidal side-effects after acute and chronic antipsychotic exposure (Lockwood and Remington, 2015). Acute antipsychotic exposure in humans results in parkinsonism, though this is often reversible with discontinued use of the antipsychotic (Thanvi and Treadwell, 2009). Wagner et al. (1988) found that acute antipsychotic-induced parkinsonism was more common in smokers. Smokers, compared to non-smokers, have higher CYP2D6 in the brain (Mann et al., 2008; Miksys and Tyndale, 2004), without differences in CYP2D6 in the liver (Bock et al., 1994; Funck-Brentano et al., 2005; Steiner et al., 1985). Similarly, rats and African Green monkeys given chronic nicotine treatment have higher CYP2D in the brain, but similar CYP2D activity in the liver, compared to controls (Mann et al., 2008; Miller et al., 2014; Yue et al., 2008). It is possible that CYP2D6 in the brain may play a role in response to short-term antipsychotic use, with increased CYP2D6 in the brain of smokers resulting in increased acute antipsychotic-induced parkinsonism, perhaps via the creation of a toxic metabolite in the brain.

Chronic antipsychotic exposure in humans results in tardive dyskinesia, a serious and often irreversible involuntary movement disorder involving the orofacial, trunk, and limb regions of the body (Lockwood and Remington, 2015; Turrone et al., 2003b). *CYP2D6* EMs, which have higher CYP2D6 in brain and liver compared to *CYP2D6* IMs and PMs, have lesser susceptibility of developing tardive dyskinesia compared to *CYP2D6* IMs and PMs (MacNeil and Muller, 2016). In addition, Winterer et al. (2010) found that chronic antipsychotic-induced tardive dyskinesia was less prevalent in smokers. Taken together, it is possible that CYP2D in the brain may play a role

in response to long-term antipsychotic use, with increased CYP2D6 in the brain of smokers resulting in decreased chronic antipsychotic-induced tardive dyskinesia, perhaps via the elimination of the toxic parent compound (i.e. antipsychotic) in the brain. This is supported by work in rats, where nicotine treatment significantly reduced haloperidol-induced vacuous chewing movements (Bordia et al., 2012), a model of tardive dyskinesia in rodents (Lockwood and Remington, 2015; Turrone et al., 2003b).

To specifically test whether variation in CYP2D activity in the brain can impact acute and chronic antipsychotic response, CYP2D in rat brain was manipulated by selective inhibition and induction, leaving CYP2D in rat liver unchanged, and the behavioural responses to acute and chronic haloperidol treatment were assessed. Selective and irreversible inhibition of CYP2D in rat brain, without inhibiting CYP2D in rat liver, has been demonstrated *in vivo* using 24-hour pre-treatment with intracerebroventricular (icv) propranolol before administration of the CYP2D-substrates codeine (McMillan and Tyndale, 2015; Zhou et al., 2013) and oxycodone (McMillan et al., 2019), and followed by the measurement of analgesia. Selective induction of CYP2D in rat brain was also demonstrated *in vivo* using daily subcutaneous (sc) injection of nicotine for 7 days, in these same models of opioid analgesia (McMillan et al., 2019; McMillan and Tyndale, 2015, 2017). However, it is unknown whether altering CYP2D in rat brain would alter brain haloperidol drug levels and haloperidol response. Furthermore, it is unknown whether CYP2D in brain can play a role in response to chronically administered drugs.

Objectives. The objectives of this study were to determine whether inhibiting and inducing CYP2D in rat brain alter brain haloperidol levels (without affecting hepatic CYP2D activity or plasma haloperidol levels) sufficiently to alter haloperidol response using well-characterized rat

models of antipsychotic-induced acute parkinsonism and chronic tardive dyskinesia (Castagne et al., 2009; Creed and Nobrega, 2013).

Study 2. **"Propranolol is a mechanism-based inhibitor of CYP2D and CYP2D6 in humanized CYP2D6-transgenic mice: effects on activity and drug response"**. In this study, the transgenic CYP2D6 mouse line (TG) that has both the mouse *Cyp2ds* and the human *CYP2D6* genes (Cheng et al., 2013) was used. Earlier work did not assess relative velocities of TG and WT CYP2D metabolism in the liver. Propranolol inhibition in TG could be a useful tool to investigate CYP2D6 *in vivo* and *ex vivo*. Propranolol has been shown to act as an MBI of human CYP2D6 in humans and rat CYP2D; however, it is not known if propranolol acts as an MBI of mouse and human CYP2D expressed in mice. This step is necessary before we can use propranolol to selectively inhibit brain CYP2Ds in TG and WT both *in vivo* and *in vitro*.

Objectives. The objectives of this study were to use TG (Cheng et al., 2013) and C57BL/6 wildtype mice (WT) 1) to determine the relative *ex vivo* velocity of hepatic CYP2D metabolism, 2) to assess *in vitro* propranolol as an MBI of CYP2D in liver microsomes from TG and WT mice and humans, and to derive inhibition and inactivation parameters, and 3) to investigate the effect of *in vivo* 24-hour pre-treatment with intraperitoneal (ip) propranolol on the *in vivo* metabolism of dextromethorphan and haloperidol (both CYP2D6 substrates) (Schmid et al., 1985; Shin et al., 2001). Haloperidol-induced catalepsy, an acute behavioural response, was also studied to assess the possible *in vivo* pharmacodynamic effects of propranolol pre-treatment on metabolism catalyzed by CYP2D.

Study 3. **"Human CYP2D6 is functional in brain *in vivo*: evidence from humanized CYP2D6-transgenic mice"**. As a first step towards translational studies of CYP2D6 *in vivo* in human brain, we explored the impact of human CYP2D6 in mouse brain *in vivo* on drug response from study 2, propranolol was found to be an MBI of human CYP2D6 (in human and TG liver) and of mouse CYP2D (in TG and WT liver). Thus, it was expected that propranolol administered into the mouse brain would also act as an MBI of both human CYP2D6 (in TG brain) and mouse CYP2D (in TG and WT brain).

Objectives. The objectives of this study were to assess *in vivo* 24-hour pre-treatment with icv propranolol as an MBI of CYP2D in TG and WT brain, by measuring the *in vivo* metabolism of dextromethorphan and haloperidol, by assessing haloperidol-induced catalepsy, and by quantifying the *ex vivo* dextrorphan formation rate by brain membranes.

Review of Literature

1-1 Cytochrome P450 2D6 Enzymes

1-1-A CYP2D6 general introduction

The cytochrome P450 (CYP) enzymes are a superfamily of heme-containing monooxygenase enzymes involved in the oxidative metabolism of a wide range of substrates (Cook et al., 2016; Nebert and Russell, 2002). CYPs catalyze a number of diverse reactions, including dehalogenations, dealkylations, deaminations, desulphurations, epoxidations, hydroxylations, N-oxide reductions, peroxidations, and sulphoxidations (Hannemann et al., 2007). Based on amino acid sequence homologies, CYPs are arranged into families (40% similarity) and subfamilies (55% similarity) (Anzenbacher and Anzenbacherová, 2001; Nebert and Russell, 2002). Most therapeutic drugs are metabolized by CYPs (Zanger and Schwab, 2013). Drug metabolizing CYPs are highly expressed in liver, where a majority of drug metabolism occurs, altering systemic drug and metabolite levels (Wrighton and Stevens, 1992). However, plasma drug levels do not always correlate with therapeutic effect, especially for centrally acting drugs (Ding and Kaminsky, 2003; Michels and Marzuk, 1993). Some CYPs, including CYP2D (referred to as CYP2D6 for human and herein as CYP2D for all other species), are also expressed and enzymatically active in brain and other extrahepatic organs, though usually at lower levels compared to liver.

Cytochrome P450 2D6 (CYP2D6) is an important drug metabolizing enzyme estimated to be involved in the oxidation of 20-30% of clinically used drugs (Yu et al., 2004; Zanger and Schwab, 2013). Using pharmacopores, the specific arrangement of small molecules used to study ligand interactions with larger macromolecules (Bajorath, 2017), common structural characteristics have been defined for CYP2D6 substrates. For example, most CYP2D6 substrates have a basic nitrogen

that is 5-7 or 10 Å from the oxidation site, a flat hydrophobic region near the oxidation site, and a negative molecular electrostatic potential above this hydrophobic area (Bonn et al., 2008; de Groot et al., 1999). CYP2D6 metabolizes a large number of exogenous compounds (Table 1); CYP2D6 metabolizes these compounds into more active forms (e.g. codeine to morphine) (Dayer et al., 1988), equally active forms (e.g. methamphetamine to amphetamine) (Lin et al., 1997), or less active forms (e.g. desipramine to 2-hydroxydesipramine) (Bertilsson and Aberg-Wistedt, 1983).

Table 1:Examples of CYP2D6 substrates

Analgesics / Antitussive	Antidepressants	Antiemetics	Antipsychotics	Neurotoxins
Bicifadine	Citalopram	Dolasetron	Aripiprazole	ß-carbolines
Codeine	Fluoxetine	Metoclopramide	Haloperidol	Diuron
Dextromethorphan	Paroxetine	Ondansetron	Perphenazine	MPTP
Oxycodone	Nortriptyline	Tropisetron	Risperidone	TIQ
Antiarrhythmics	Antiestrogen	Antihistamines	ß-adrenergic blockers	Recreational drugs
Cibenzoline	Droloxifene	Azelastine	Bufuralol	Amphetamine
Encainide	Enclomifene	Diphenhydramine	Metoprolol	MDA
Propafenone	Lasofoxifene	Epinastine	Propranolol	MDMA
Sparteine	Tamoxifen	Mequitazine	Timolol	MPBP

MDA, methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MPBP, 4´-methyl-alpha-pyrrolidinobutyrophenone; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TIQ, tetrahydroisoquinoline. Adapted from Zhou 2016.

The drugs listed in Table 1 are metabolized by CYP2D6. Some of these drugs are probe substrates while many are also metabolized by other CYPs; the velocities and binding affinities may differ between the different CYPs involved in their metabolism. According to the Food and Drug Administration (FDA), "a probe substrate should be selective (e.g. predominantly metabolized by a single enzyme in pooled human liver microsomes or recombinant CYPs) and have simple metabolic schemes" (FDA, 2020). The ratio of a metabolite concentration to the parent probe drug concentration can act as a phenotypic marker of the activity of the CYP of interest (of note, the parent over the metabolite has also been used historically). Typical probe drugs for CYP2D6 include dextromethorphan, debrisoquine, sparteine, and metoprolol (Frank et al., 2007; Streetman et al., 2000). CYP2D6 is a major pathway for dextromethorphan metabolism (Küpfer et al., 1986; Schmid et al., 1985); the majority of dextromethorphan is metabolized by CYP2D6 to dextrorphan (Figure 1).

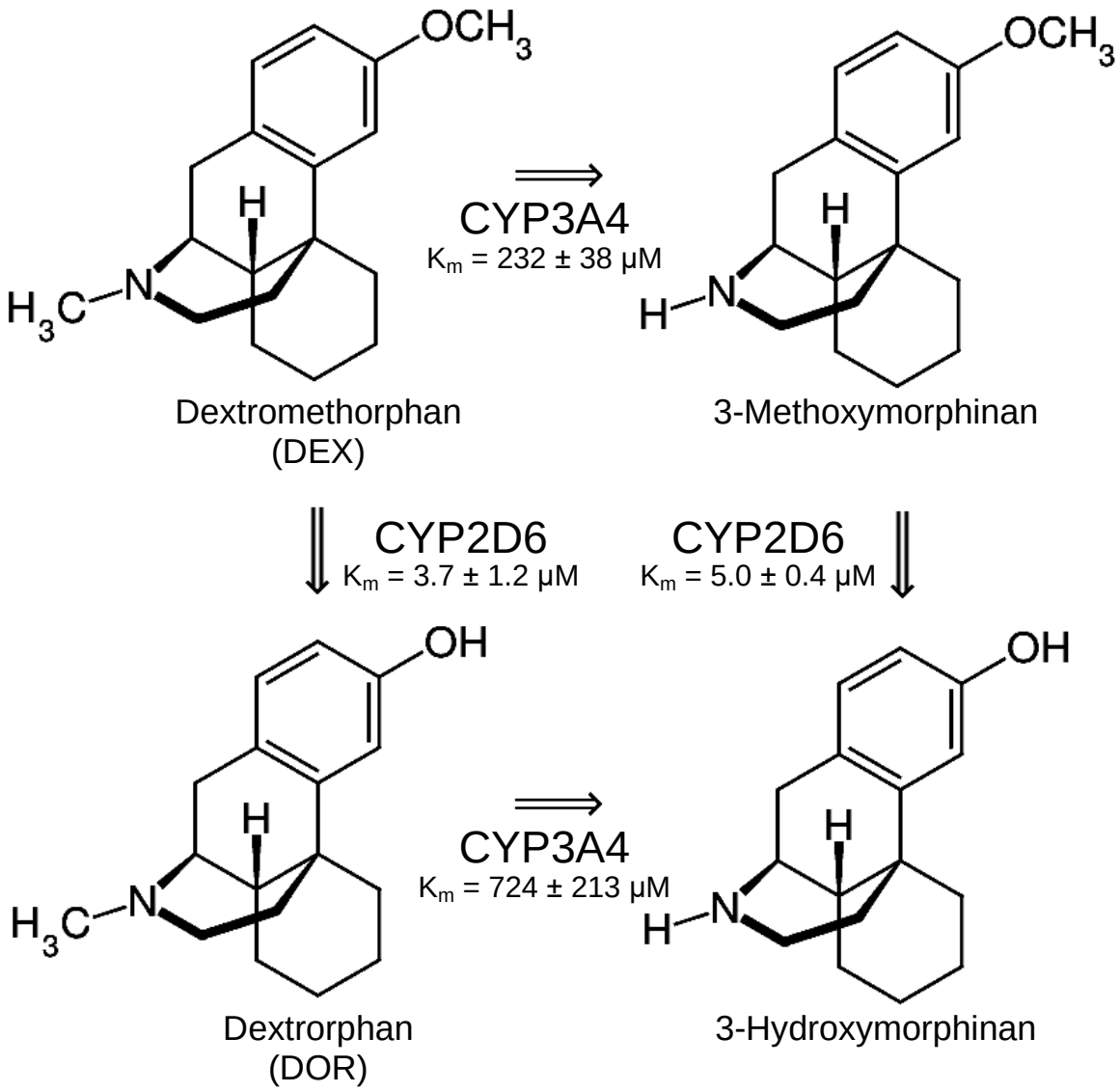


Figure 1. Human CYP2D6 and CYP3A4 metabolize dextromethorphan via demethylation pathways*. Kms listed are based on data from recombinant human CYP2D6 and CYP3A4. Adapted from Yu and Haining 2001.*

1-1-B CYP2D6 inhibitors

Inhibitory compounds typically result in reversible and time-dependent inhibition (e.g. competitive, non-competitive, and uncompetitive inhibitors) (Segel, 1975); however, some compounds result in irreversible and time-independent inhibition (Silverman, 1995). Competitive inhibitors reversibly bind to, or near, the CYP catalytic site, blocking the metabolism of other

substrates (Segel, 1975). A CYP substrate can act as a competitive inhibitor of metabolism of a second substrate by the same CYP. Non-competitive inhibitors reversibly bind to the enzyme at a site that may be distant to the CYP catalytic site; binding of a non-competitive inhibitor results in changes in the enzyme conformation, preventing access of substrates to the catalytic site (Segel, 1975). Uncompetitive inhibitors bind reversibly to an enzyme-substrate complex and form an inactive enzyme-substrate-inhibitor complex (Segel, 1975). Mechanism-based inhibitors (MBIs), also referred to as irreversible or suicide inhibitors, are substrates that are metabolized to reactive intermediates that then covalently bind to the enzyme, rendering it irreversibly inactivated (Silverman, 1995). MBIs result in inhibition that is relatively long lasting, with activity returning following new enzyme synthesis (Liston et al., 2002; McDonald and Tipton, 2011). Examples of CYP2D6 inhibitors are listed in Table 2.

Table 2: Examples of CYP2D6 inhibitors

	Reversible Inhibitors		Mechanism-Based Inhibitors
Antiarrhythmics	Propafenone	Quinidine	Desethylamiodarone
Antidepressants	Citalopram	Desipramine	Paroxetine
	Duloxetine	Fluoxetine	
	Imipramine	Nortriptyline	
Antiemetics			Metoclopramide
Antihistamine	Azelastine	Mepyramine	Cimetidine
Antipsychotics	Clozapine	Haloperidol	Pimozide
	Risperidone	Thioridazine	
ß-adrenergic blockers	Bufuralol	Metoprolol	
	Oxprenolol	Timolol	
Recreational Drugs	Amphetamine	MDA	MDMA

MDA, methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine. Adapted from Rendic 2002 and Zhou 2016.

Propranolol is a β-adrenoceptor antagonist metabolized by CYP2D6 (Lennard et al., 1984). In addition to being a substrate it is thought to be a CYP2D6 MBI, requiring enzyme-catalyzed metabolism for irreversible inhibition to occur in human liver microsomes (Shaw et al., 1987). Propranolol is also an MBI of rat CYP2D in liver microsomes (Masubuchi et al., 1991; Schneck and Pritchard, 1981). *In vivo* propranolol is also believed to be an MBI of CYP2D in human (Rowland et al., 1994) and rat (Schneck and Pritchard, 1981) liver. Rowland et al. (1994) found

evidence that *in vitro* 4-hydroxypropranolol, a propranolol metabolite, may be an MBI of CYP2D6 in human liver microsomes; however, they did not test propranolol itself as an MBI of CYP2D6. While propranolol is also metabolized by CYP3A4 and CYP1A2, it does not appear to be an MBI for these enzymes (Herman et al., 1983; Walle et al., 1987). Except for propranolol, we could find no evidence that other β-adrenoceptor antagonists are suspected to be MBIs of CYP2D6 or other enzymes.

1-1-C CYP2D6 genetic variation

The *CYP2D6* gene is highly genetically polymorphic with numerous *CYP2D6* allelic variants. *CYP2D6* PMs are individuals with homozygous or compound heterozygous constellations of null alleles (alleles that do not encode a functional protein), *CYP2D6* IMs are individuals typically with one reduced function and one null allele, *CYP2D6* EMs are individuals with two functional alleles, and *CYP2D6* UMs are individuals with gain-of-function variants (Figure 2) (Gaedigk, 2013; Gaedigk et al., 2008; Zanger et al., 2004; Zanger and Schwab, 2013). *CYP2D6* PMs have little to no CYP2D6 enzyme activity (Eichelbaum et al., 1979; Mahgoub et al., 1977) and their *CYP2D6* genetic status has been associated with greater side effects when taking antineoplastic drugs (Jung and Lim, 2014; Zeng et al., 2013), antidepressants (de Leon et al., 2005; Llerena et al., 2004), and antipsychotics (Brockmöller et al., 2002; Schillevoort et al., 2002). *CYP2D6* PMs also experience little analgesia after taking opioids that require metabolic activation by CYP2D6, such as codeine (Zahari and Ismail, 2014).

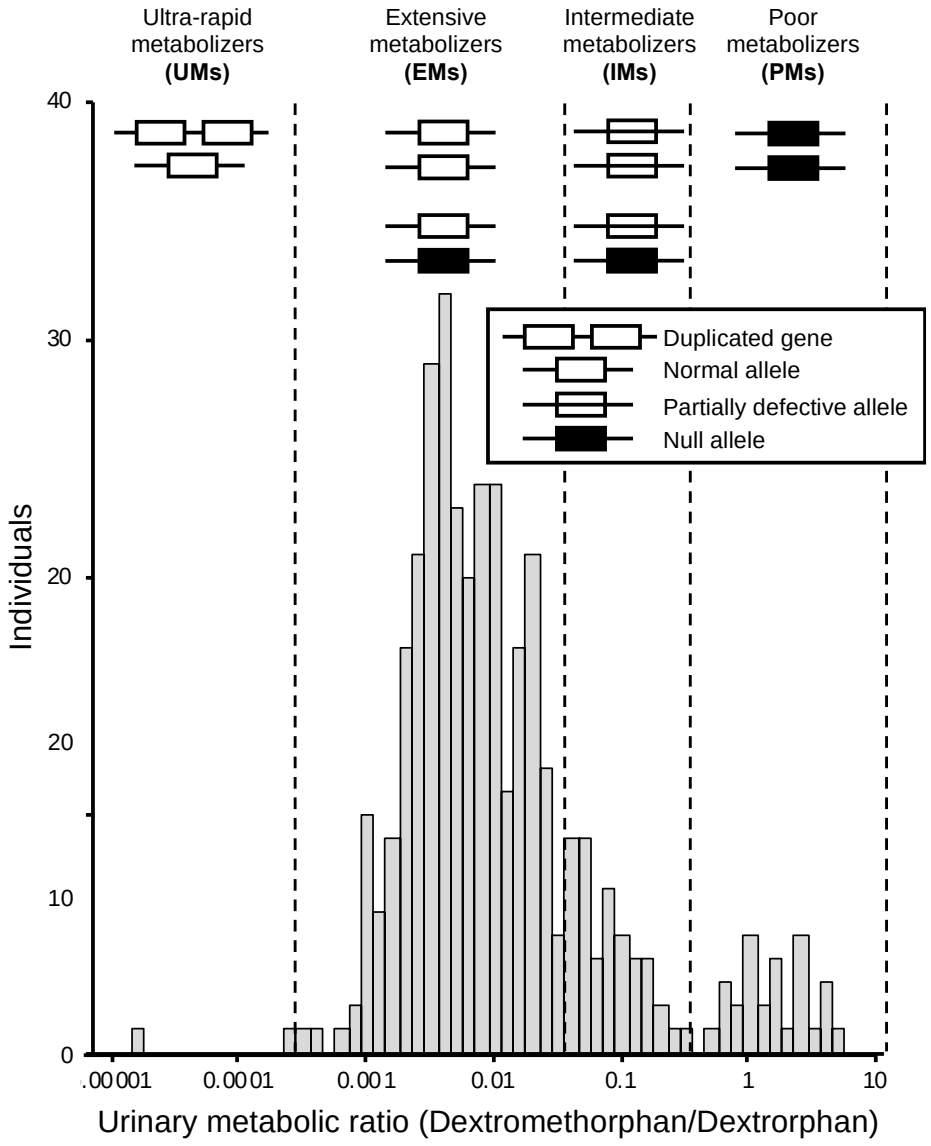


Figure 2. Frequency histogram of CYP2D6 activity and genotype distribution from a cohort of 362 Caucasian subjects*. The ratio of a metabolite concentration to the parent probe drug concentration, or as shown here the ratio of parent probe drug concentration to the metabolite concentration, is a phenotypic marker of CYP activity. In this case, CYP2D6 activity is measured by the urinary ratio of the parent dextromethorphan to its metabolite dextrorphan; this ratio is used to classify this population into ultra-rapid metabolizers, extensive metabolizers, intermediate metabolizers, and poor metabolizers. Adapted from Gaedigk et al., 2008.*

1-1-D CYP2D6 non-genetic variation

Levels of CYP2D6 in the liver fluctuate very little throughout a person's life (Treluyer et al., 1991).

In addition, CYP2D6 in liver is considered uninducible; typical CYP inducers like rifampicin and

phenobarbital do not result in induction of CYP2D6 in human liver slices (Edwards et al., 2003) and in cultured human hepatocytes (Madan et al., 2003), and thus CYP2D6 in the liver is regulated primarily by genetics. CYP2D6 in the liver is subject to inhibition from a large variety of compounds (see Table 2). Phenoconversion is the process by which a genotype-predicted enzymatic activity is converted to a different phenotype, typically due to enzymatic inhibition (Shah and Smith, 2015; Zanger and Schwab, 2013). Drugs such as methylenedioxymethamphetamine (Heydari et al., 2004), paroxetine (Bertelsen et al., 2003), or cimetidine (Madeira et al., 2004) are known to be MBIs of CYP2D6 leading to irreversible inhibition of CYP2D6 and the phenoconversion of *CYP2D6* UMs and EMs to *CYP2D6* IMs/PMs (Juřica and Žourková, 2013; O'Mathúna et al., 2008).

Pregnancy is associated with dynamic anatomical, biochemical, and physiological changes in the maternal body, which can result in changes to pharmacokinetics and pharmacodynamics of clinically used drugs (Gaohua et al., 2012; Tracy et al., 2005). CYP activity can increase (CYP2A6, CYP2C9, CYP2D6, and CYP3A4) or decrease (CYP1A2 and CYP2C19) during the gestational period (Anderson, 2005). Pregnant women have induced CYP2D6 metabolism in the liver, as demonstrated by increased clearance of clonidine (Buchanan et al., 2009; Claessens et al., 2010), dextromethorphan (Tracy et al., 2005; Wadelius et al., 1997), fluoxetine (Heikkinen et al., 2003), metoprolol (Högstedt et al., 1985; Högstedt et al., 1983), nortriptyline (Wisner et al., 1993), and paroxetine (Ververs et al., 2009). While it is possible that elevated estrogen and/or progesterone during pregnancy induces human CYP2D6, we have found no further evidence to support this hypothesis (albeit difficult to generate). Consistent with the interpretation of induction during pregnancy, the increases in CYP2D6 activity were not observed in *CYP2D6* PMs. For example, Ververs et al. (2009) observed that, compared to non-pregnant women of the same

CYP2D6 genotype, pregnant women who were *CYP2D6* EMs had increased dextromethorphan metabolism whereas pregnant women who were *CYP2D6* PMs did not.

1-2 CYP2D in rodents

CYP2Ds have been categorized and named in numerous animals, including in rats (Hiroi et al., 1998a; Komori, 1993) and mice (Miksys et al., 2005). Humans have only one functional isozyme of CYP2D (i.e. CYP2D6), whereas rats have six (CYP-2D1, -2D2, -2D3, -2D4, -2D5, -2D18), and mice have nine (CYP-2D9, -2D10, -2D11, -2D12, -2D13, -2D22, -2D26, -2D34, and -2D40) (Martignoni et al., 2006). The rat CYP2D thought to be orthologous to CYP2D6 is the CYP2D1 enzyme (Martignoni et al., 2006), while the mouse CYP2D thought to be orthologous to CYP2D6 is the CYP2D22 enzyme (Blume et al., 2000; Martignoni et al., 2006). Despite having similar amino acid sequences, CYP2D isozymes vary slightly from each other in substrate specificity and enzymatic activity profiles. For example, the human CYP2D6 inhibitor quinidine (Nedelcheva and Gut, 1994; Strobl et al., 1993) is a less potent inhibitor of rat CYP2D (Kobayashi et al., 1989) while quinine, quinidine's diastereomer, is a more potent inhibitor of rat CYP2D, compared to human CYP2D6 (Kobayashi et al., 1989). Isozymes of rat CYP2D exhibit differential tissue expression, for example rat CYP2D1 and CYP2D2 are abundantly expressed in the liver (Hiroi et al., 1998a; Wyss et al., 1995), whereas rat CYP2D4 is primarily expressed in the brain (Hiroi et al., 1998a; Komori, 1993; Wyss et al., 1995). There are fewer reports of differential tissue expression of mouse CYP2D isozymes, although there has been some interest in CYP2D22 due to its amino acid similarity to human CYP2D6. Mouse CYP2D22 was found to be abundantly expressed in the liver, as well as in other organs such as adrenal, ovary, and mammary glands, and in the brain (Blume et al., 2000).

To investigate human CYP2D6 drug interactions and drug metabolism, a transgenic *CYP2D6* mouse line, which included the complete human *CYP2D6* gene and its regulatory sequence, was created (Corchero et al., 2001). This was done to minimize issues of CYP2D-isozyme and species differences that have been observed (Corchero et al., 2001; Shen et al., 2007). These transgenic mice expressed mouse CYP2D isozymes and the human CYP2D6 in the liver, kidney, and intestines, but did not express CYP2D6 in the brain (Miksys et al., 2005). Scheer et al. (2012) developed a number of mouse lines of interest, including *Cyp2d* cluster knockout mice that contained homozygous deletions of the nine full length mouse *Cyp2d* genes and a different CYP2D6-humanized transgenic mice than the one used here, that had the human *CYP2D6* gene while also being devoid of the nine mouse *Cyp2d* genes. This latter mouse line expressed transgenic CYP2D6 in the liver, kidneys, and intestines, and metabolized known CYP2D6 substrates bufuralol and debrisoquine, but the expression of CYP2D6 in the brain was not determined (Scheer et al., 2012). More recently, another transgenic mouse line (TG) was created with a wider CYP2D6 tissue distribution, including in the brain, and with tissue-specific regulation of the human *CYP2D6* transgene (Cheng et al., 2013). These TG mice can model overexpression of CYP2D since they have both the mouse *Cyp2ds* and the human *CYP2D6* genes. This enables the safe investigation of the role of human CYP2D6 in drug interactions, drug responses, toxicity, therapeutic effect, drug reward, and/or risk of abuse by allowing the manipulation of human CYP2D6 activity (e.g. inhibited) in a controlled manner in animals (versus in people).

1-3 CYP2D in brain

1-3-A CYP2D substrates in the brain

Of note, CYP2D6 metabolizes a large number of clinical substrates that act in the brain, such as antipsychotics (e.g. haloperidol), antidepressants, and opioids (Ingelman-Sundberg, 2005; Zanger

and Schwab, 2013) (Table 3). In addition, endogenous ligands, such as trace amines (e.g. tyramine to dopamine (Hiroi et al., 1998b) and 5-methoxytryptamine to serotonin (Yu et al., 2003a)) and neurosteroids (e.g. progesterone (Hiroi et al., 2001)), are metabolized by CYP2D6. CYP2D6 can also metabolize neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Coleman et al., 1996), which can induce Parkinson-like symptoms (Langston et al., 1983). Thus, variable CYP2D6-mediated drug metabolism specifically within brain may alter brain toxin/drug and metabolite levels and have a resulting impact on the therapeutic or neurotoxic effects (Krishna and Klotz, 1994).

Table 3: Examples of centrally acting CYP2D6 substrates

Tricyclic antidepressants	Other antidepressants	Opioids
Amitriptyline	Duloxetine	Codeine
Desipramine	Escitalopram	Hydrocodone
Imipramine	Mirtazapine	Oxycodone
Nortriptyline	Venlafaxine	Tramadol
SSRIs	Antipsychotics	Antiemetics
Citalopram	Aripiprazole	Dolasetron
Fluoxetine	Haloperidol	Metoclopramide
Paroxetine	Perphenazine	Ondansetron
Sertraline	Risperidone	Tropisetron

SSRI, Selective serotonin reuptake inhibitor. Adapted from Zhou 2016.

1-3-B Expression of CYP2D in the brain

CYP2D is expressed in the brain and has been identified in the brains of rats (Fonne-Pfister et al., 1987; Tyndale et al., 1999), mice (Miksys et al., 2005), dogs (Niznik et al., 1990), monkeys (Mann et al., 2008; Miller et al., 2014), and humans (Fonne-Pfister et al., 1987; Miksys et al., 2005; Siegle et al., 2001; Tyndale et al., 1991). The presence of CYP2D6 in human brain has been demonstrated using a variety of techniques, including Western blotting (Fonne-Pfister et al., 1987), cDNA cloning (Tyndale et al., 1991), *in vitro* phenotyping via dextromethorphan (Voirol et al., 2000), and *in situ* hybridization coupled with immunohistochemistry (Siegle et al., 2001). CYP2D6 is expressed to a different extent among brain regions, as assessed in the cerebellum, the frontal cortex, the globus pallidus, the hippocampus, the nucleus accumbens, the putamen, and the substantia nigra (Miksys et al., 2002; Siegle et al., 2001). CYP2D6 expression has also been found to be cell-type specific, including for example expression in the pyramidal cells of the cortex and hippocampus, Purkinje and glial cells of the cerebellum, and pigmented neurons of the substantia nigra (Dutheil et al., 2009; Siegle et al., 2001).

The *ex vivo* activity of CYP2D in brain tissue has been measured in rats using multiple probe substrates, including the hydroxylation of bufuralol (Coleman et al., 2000), the demethylation of codeine (Chen et al., 1990), dextromethorphan (Voirol et al., 2000), and 3-methylenedioxymethamphetamine (Lin et al., 1992), and in dogs using the demethylation of sparteine (Tyndale et al., 1991). The substrate affinities (K_m) to CYP2D in rat brain are similar to substrate affinities to CYP2D in rat liver; however, the maximal substrate turnover rate (V_{max}) is lower in rat brain, as expected due to lower CYP2D expression, compared to rat liver (Coleman et al., 2000; Tyndale et al., 1999). *Ex vivo* dextromethorphan *O*-demethylation to dextrorphan differs across rat brain regions, with the cerebellum having the highest CYP2D activity (Tyndale et al.,

1999). This higher CYP2D activity is concordant with the higher expression of CYP2D enzyme in rat cerebellum, compared to other brain regions, and the strong correlations between CYP2D activity and CYP2D enzyme expression across brain regions (Miksys et al., 2000).

Human CYP2D6 in the liver is considered uninducible and is regulated solely by genetics (Edwards et al., 2003; Madan et al., 2003), whereas CYP2Ds in the brain can be readily induced by drugs (Mann et al., 2008; Miksys and Tyndale, 2013; Yue et al., 2008). For example, it was found in human post-mortem studies that alcoholics have higher CYP2D6 in the brain compared to non-alcoholics (Miksys et al., 2002; Miksys and Tyndale, 2004), with no difference in levels of CYP2D6 in the liver (Steiner et al., 1985). Smokers also have higher CYP2D6 in the brain compared to non-smokers (Mann et al., 2008; Miksys and Tyndale, 2004), with no difference in levels of CYP2D6 in liver (Bock et al., 1994; Funck-Brentano et al., 2005; Steiner et al., 1985). Daily sc nicotine, vs saline, treatment for seven days induced CYP2D in the brain of rats, including in the striatum, cerebellum, and hippocampus; there was no difference in levels of CYP2D in the liver (Yue et al., 2008). This chronic nicotine treatment resulted in the highest increase of CYP2D levels in the brain eight hours after the last nicotine injection (Yue et al., 2008); at this time there was no detectable plasma nicotine (Micu et al., 2003). CYP2D levels in the rat brain return to baseline approximately twelve hours after the last nicotine injection (Yue et al., 2008). Chronic nicotine (Mann et al., 2008; Miller et al., 2014) or alcohol (Miller et al., 2014) treatment also induced CYP2D in the brain of African Green monkeys.

1-3-C Functional impact of CYP2D variation in the brain

CYP2D is metabolically active in animal brains where it can influence local drug and metabolites levels sufficiently to alter drug response (Miksys and Tyndale, 2013). A useful method to study the *in vivo* role of CYP2D in brain drug metabolism and response involves the

central administration of inhibitors (such as propranolol) to animals to inhibit CYP2D in brain, or chronic sc administration of nicotine to induce CYP2D in brain, with both methods having no impact on hepatic CYP2D. For example, manipulation of CYP2D in rat brain can alter codeine analgesia, which requires CYP2D-mediated activation of codeine to morphine (McMillan and Tyndale, 2015; Zhou et al., 2013). While a role has been demonstrated for CYP-mediated metabolism in rat brain on drug response *in vivo* (McMillan et al., 2019; McMillan and Tyndale, 2015), it is unclear if human CYP2D6 can function within brain and contribute meaningfully to central drug metabolism and response.

1-4 Haloperidol

Haloperidol is a CYP2D6 substrate used in the treatment of schizophrenia (Shin et al., 2001). Schizophrenia has a world-wide prevalence of approximately 0.48% (Simeone et al., 2015), frequently requiring life-time management with antipsychotic drugs. These drugs act primarily through striatal dopamine receptor D_2 blockade and can cause severe extra-pyramidal side-effects after acute and chronic exposure (Lockwood and Remington, 2015). Newer, atypical drugs have lower risk for side-effects, but have a higher potential for metabolic side-effects (Lockwood and Remington, 2015). The underlying pathophysiology of side-effects is not clear and may differ after acute and chronic antipsychotic treatment. For example, acute haloperidol treatment can cause parkinsonism, which is reversible on cessation of treatment and long term haloperidol treatment can cause tardive dyskinesia, a debilitating motor side-effect which is rarely reversible (Lockwood and Remington, 2015).

Many antipsychotics, including typicals and atypicals, are metabolized by the CYP2D6 enzyme (Pouget et al., 2014). In humans *CYP2D6* genotype is associated with plasma levels of some antipsychotics where *CYP2D6* PMs have higher plasma drug levels and are considered at higher

risk for tardive dyskinesia compared to normal metabolizers with the same dosing (van der Weide and van der Weide, 2015). The relationship between *CYP2D6* genotype and acute parkinsonism is less clear. Some metabolites of antipsychotics that are produced by CYP2D6 are potentially neurotoxic (Subramanyam et al., 1991; Subramanyam et al., 1990), but the relative roles of the parent and metabolites in side-effects are unknown. While brain striatal dopamine receptor D_2 occupancy and resulting antipsychotic efficacy are closely related, they are not necessarily related to antipsychotic plasma levels (Tauscher et al., 2002), and this disconnect, taken with other risk factors for tardive dyskinesia, such as gender, age, and length of illness, make it difficult to identify sources of inter-individual variation in risk for side-effects. Therefore, individuals with similar CYP2D activity in liver and antipsychotic levels in plasma may have higher or lower CYP2D activity in brain resulting in variation of antipsychotics levels in brain (and CYP2D-mediated metabolites) and striatal dopamine receptor D_2 occupancy. These differences in brain drug and metabolite levels could contribute to the variation in risk for parkinsonism and tardive dyskinesia in patients taking antipsychotics.

Haloperidol is used acutely to treat a variety of conditions including nausea (Murray-Brown and Dorman, 2015), delirium (Siddiqi et al., 2016), aggressive behaviour (Powney et al., 2012), migraine (Gaffigan et al., 2015), cannabinoid hyperemesis (Jones and Abernathy, 2016) and obsessive compulsive disorder (McDougle et al., 1994). Chronic haloperidol is widely used to manage schizophrenia, especially in less wealthy countries, and is on the World Health Organization's list of essential drugs (World Health Organization, 2019). Antipsychotics have been well characterized in animal models of acute and chronic side-effects. Catalepsy is an acute response to first generation antipsychotics, including haloperidol, and is used to predict the likelihood of a drug (e.g. an antipsychotic) causing extrapyramidal side effects in humans (Hoffman and Donovan, 1995). It can also be considered a model of acute antipsychotic-induced

parkinsonism (Turrone et al., 2003b). Vacuous chewing movements in rodents are a response to chronic exposure to antipsychotics, including haloperidol, and are a model of the human adverse response to chronic exposure to antipsychotics and of tardive dyskinesia (Lockwood and Remington, 2015; Turrone et al., 2003b).

1-5 Statement of Research Hypotheses

In **study 1,** "**CYP2D enzymatic metabolism in the rat brain alters acute and chronic haloperidol side-effects by different mechanisms"**, we hypothesized that (1) an increase in haloperidol metabolism within the brain will increase acute haloperidol-induced catalepsy, and (2) a decrease in haloperidol metabolism within the brain will decrease acute haloperidol-induced catalepsy and increase chronic haloperidol-induced vacuous chewing movements.

In **study 2, "Propranolol is a mechanism-based inhibitor of CYP2D and CYP2D6 in humanized CYP2D6-transgenic mice: effects on activity and drug response"**, we hypothesized the following: (1) TG will have a higher *ex vivo* dextrorphan formation rate in the liver compared to WT. (2) *In vitro* propranolol will act as an MBI of CYP2D, resulting in a decreased *in vitro* dextrorphan formation rate. (3) *In vivo* 24-hour pre-treatment with ip propranolol will act as an MBI of CYP2D *in vivo*, resulting in decreased *in vivo* plasma dextrorphan/dextromethorphan ratios and a decreased *ex vivo* dextrorphan formation rate in both TG and WT. (4) *In vivo* 24-hour pre-treatment with ip propranolol will act as an MBI of CYP2D *in vivo*, resulting in higher plasma haloperidol levels and higher haloperidol-induced catalepsy in both TG and WT.

In **study 3, "Human CYP2D6 is functional in brain *in vivo*: evidence from humanized CYP2D6-transgenic mice"**, we hypothesized the following: (1) After 24-hour pre-treatment with icv vehicle, TG will have a higher *ex vivo* dextrorphan formation rate in the brain compared to WT. (2) *In vivo* 24-hour pre-treatment with icv propranolol will act as an MBI of CYP2D in the brain, resulting in a decrease in *ex vivo* dextrorphan formation rate in the brain of WT and an even greater decrease in TG, and resulting in a decrease in the *in vivo* dextrorphan/dextromethorphan ratio in the brain of WT and an even greater decrease in TG. (3) *In vivo* 24-hour pre-treatment with icv propranolol will act as an MBI of CYP2D in the brain *in vivo*, resulting in decreased

haloperidol-induced catalepsy in WT and an even greater decrease in TG. (4) *In vivo* 24-hour pre-treatment with icv propranolol will only inhibit brain CYP2D, therefore the *ex vivo* dextrorphan formation rate in the liver, the *in vivo* dextrorphan/dextromethorphan ratio in plasma, and the *in vivo* haloperidol levels in plasma will not be different compared to *in vivo* 24-hour pre-treatment with icv vehicle.

Initial experiments revealed that *in vivo* propranolol given icv acts as an MBI of CYP2D6 in TG brain, but not of mouse CYP2D in WT brain (see results in Section 3-3). Although unexpected, this provided a novel strategy to selectively study human CYP2D6 in the brain (leaving mouse CYP2Ds in the brain and liver, and CYP2D6 in the liver unaffected) in a live animal. This provided a model for evaluating the role of CYP2D6 *in vivo* in the mammalian brain. While studies in humans have associated genetic variation in *CYP2D6* with differences in neurobiological function, this CYP2D6 transgenic mouse model could provide a method for directly testing the roles of CYP2D6 in the brain. Using this model, it was demonstrated that human CYP2D6 in the brain is active *in vivo* and *in vitro*; furthermore, it was demonstrated that there is sufficient CYP2D6 activity in the brain to alter haloperidol-induced catalepsy in mice *in vivo*. These data indicate that, within this mouse model, human CYP2D6 expressed in the brain is functional *in vivo* and contributes to drug response. In addition, this model provides a novel tool to understand better the role that CYP2D6 in brain plays in drug response, neurotoxicity, and endogenous neurotransmitter metabolism.

2 Methods

2-1 Study 1: CYP2D enzymatic metabolism in the rat brain alters acute and chronic haloperidol side-effects by different mechanisms

2-1-A Animals and housing conditions

Adult male Wistar rats, average weight 375 g (Charles River, St. Constant, Canada), were housed in groups of 3 under a 12-hour light/dark cycle. Procedures were conducted in the light phase.

2-1-B Rat icv cannulation surgery

Rats were anesthetized with isoflurane (5% induction, 1-2% maintenance) and positioned in a stereotaxic frame. Stainless steel guide cannulas (28 ga.) were implanted into the right lateral cerebral ventricle for icv injections, with bregma coordinates dorsal-ventral 3.6 mm, lateral-medial -1.4 mm, and anterior-posterior -0.9 mm (Paxinos and Watson, 1986). Rats were given the analgesic ketoprofen (3 mg/kg sc) intraoperatively and daily for 3 days after surgery. They were allowed to recover for at least one week prior to testing. Cannula patency was maintained by sterile dummy cannulas, removed only for drug administration.

2-1-C Acute haloperidol and catalepsy

After a 1-minute acclimatization, the latency to remove a rat's forepaws from a surface raised 9 cm above the cage floor was recorded, with a 180 second cut-off. To examine the dose-response relationship, sc injections of 0.03 to 0.5 mg/kg haloperidol (injectable formulation, 5 mg/ml base in water adjusted to pH 3-3.8 with lactic acid, Omega, Montreal, Canada; n=2-5 per dose) was administered and catalepsy was assessed before (baseline, time 0) and at 30, 60, 90, and 110

minutes after injection. Brain and plasma were collected at 15, 30, 60, 90 and 110 minutes after 0.125 mg/kg sc haloperidol to assess drug levels (n=4 per time point).

2-1-D Effect of inhibiting and inducing CYP2D in the brain on acute haloperidol treatment

To test how inhibition of CYP2D in brain affects acute haloperidol-induced catalepsy, rats received *in vivo* icv injections of either propranolol hydrochloride (Sigma-Aldrich Canada, Oakville, Canada), 20 µg base (approximately 0.05 mg/kg for an average 400 g rat) in 4 µl of vehicle (sterile 40% aqueous 2-hydroxypropyl-β-cyclodextrin, Sigma-Aldrich Canada) (n=12) or 4 µL of vehicle alone (n=14) 24 hours prior to 0.125 mg/kg sc haloperidol. Catalepsy was assessed at baseline (0 minutes, i.e. immediately before haloperidol was administered) and at 30, 60, and 90 minutes after haloperidol injection. As whole brain elimination half-life of propranolol in Wistar rats is 60-70 minutes (Elghozi et al., 1979), by 24 hours propranolol has been cleared from brain and should have no central effects other than irreversible inhibition of CYP2D in brain. This was observed previously in rats tested for baseline and opioid-induced analgesia (McMillan et al., 2019; McMillan and Tyndale, 2015). Brains, livers and plasma from *in vivo* drug pre-treated rats were collected immediately following catalepsy testing to assess haloperidol levels, as described in Section 2-1-E, and to assess *ex vivo* CYP2D activity, as described in Section 2-1-F.

To test how induction of CYP2D in brain affects acute haloperidol-induced catalepsy, rats received daily sc injections of nicotine (Sigma-Aldrich, Oakville, Canada; 1 mg/kg base in sterile saline adjusted to pH 7.4) (n=23) or vehicle (n=22) for 7 days (Yue et al., 2008). Catalepsy was tested 8 hours after the last nicotine injection, when induction of CYP2D in brain is maximal (Yue et al., 2008), and nicotine has been cleared from both the brain and peripheral system (Craig et al., 2014). Catalepsy was assessed at baseline (0 minute, before haloperidol), 30, 60 and

90 minutes after 0.125 mg/kg sc haloperidol. Brains, livers and plasma from *in vivo* drug pre-treated rats were collected immediately following catalepsy testing to assess haloperidol levels, as described in Section 2-1-E, and to assess *ex vivo* CYP2D activity, as described in Section 2-1-F.

To test how subsequent inhibition of induced CYP2D in brain affects acute haloperidol-induced catalepsy, four groups of rats (n=20 per group) were pre-treated daily for 7 days with either nicotine or saline sc. On day 6, the rats received a single icv injection of *in vivo* propranolol or vehicle icv injection, the seventh sc nicotine injection was administered 16 hours after the icv injection, and haloperidol (0.125 mg/kg sc) was injected 24 hours after the icv injection and 8 hours after the seventh nicotine injection. Catalepsy was assessed 60 minutes post-haloperidol injection. Brains, livers and plasma were collected immediately following catalepsy testing to assess haloperidol levels, as described in Section 2-1-G, and to assess *ex vivo* CYP2D activity, as described in Section 2-1-H.

2-1-E Chronic haloperidol and vacuous chewing movements

Animals received intramuscular (im) injections of 7 mg/kg haloperidol decanoate (depot formulation 100 mg/ml in sesame oil with benzyl alcohol (1.2% v/v), Sandoz, Boucherville, Canada) every 21 days for 15-16 weeks. This dosing paradigm has been shown to produce vacuous chewing movements more reliably than daily injections or osmotic pump delivery of haloperidol (Turrone et al., 2003a), and more closely models the steady plasma levels observed with chronic haloperidol dosing in humans. This dose, corresponding to 0.33 mg/kg/day, is in the mid-range of the reported dose-response curve (Turrone et al., 2003b). After 2 minutes acclimatization on a 25 cm diameter stage raised 40 cm above the table, vacuous chewing

movements, defined as either single or bursts of purposeless jaw movements, were counted over 2 minutes with the aid of a mirror behind the stage.

2-1-F Effect of inhibiting CYP2D in the brain on chronic haloperidol treatment

To test how inhibition of CYP2D in brain affects chronic haloperidol-induced vacuous chewing movements, rats (n=9 per pre-treatment group) received three icv injections of either propranolol or vehicle pre-treatment every week, and all animals received im injections of haloperidol every 21 days, beginning 24 hours after the first icv injection. Vacuous chewing movements were assessed 24 hours after icv injections, twice per week and averaged for data analysis. Saphenous vein blood was drawn over the first 21 days to assess haloperidol levels. Due to loss of cannula patency, final group sizes were n=5 for propranolol and n=4 for vehicle. Baseline vacuous chewing movements were assessed prior to the first *in vivo* icv propranolol injection, and 24 hours after icv propranolol immediately prior to the first haloperidol injection. Brains, livers and plasma from *in vivo* drug pre-treated rats were collected immediately following the last behavioural test to assess haloperidol levels, as described in Section 2-1-G, and to assess *ex vivo* CYP2D activity, as described in Section 2-1-H.

In the replication study, two groups of rats (n=16 per pre-treatment group) were treated chronically with icv pre-treatments and im haloperidol as above, and saphenous vein blood was drawn every three weeks, beginning 7 days after the first haloperidol injection. After 16 weeks, animals were sacrificed and brains, livers, and plasma were collected for biochemical analyses, 7 days after the final haloperidol decanoate injection, 1 day after the last vacuous chewing movement assessment, and 24 hours after the last icv inhibitor injection. Brains, livers and plasma were collected to assess haloperidol levels, as described in Section 2-1-E, to assess *ex vivo* CYP2D activity, as described in Section 2-1-H, and to assess lipid peroxidation as described

in Section 2-1-I. Final group sizes were n=12 for propranolol (n=1 rat removed from analysis over the treatment duration due to loss of cannula patency, and n=3 rats excluded as non-responders, i.e. animals demonstrating less than 8 vacuous chewing movements in 2 minutes for the last 4 weeks of testing) and n=9 for vehicle (n=7 rats were removed over the treatment duration due to loss of cannula patency). There was no difference in animal weights between *in vivo* 24-hour icv propranolol- and vehicle-treated groups before and after haloperidol treatment. Average vacuous chewing movements over the last 4 weeks (fourth month) were used for data analyses.

2-1-G Haloperidol levels

Brain samples were prepared and analyzed by liquid chromatography-mass spectrometry (LCMS) as previously described with some modifications (Zhang et al., 2007b). Briefly, rat brains were homogenized in deionized water (1:2 w/v), centrifuged at 12,500 g for 20 min, and the supernatant was collected and stored at -80 °C until analysis. Plasma samples were prepared and analyzed by LCMS as previously described with some modifications (Zhang et al., 2007a). The internal standard, 12.5 ng haloperidol-d_4 in 5 mM ammonium formate-acetonitrile solution (30:70 v/v), was added to each sample and haloperidol calibration standards.

Compounds were resolved at ambient temperature on an Agilent 1260 HPLC equipped with a ZORBAX SB-C18 column (1.8 µm, 50 x 2.1 mm, Agilent Technologies) connected to an Agilent 6430 QQQ. Elution conditions used solvent A (5 mM ammonium formate pH 5.5 in water) and solvent B (acetonitrile). Precursor to product ion transitions were monitored with a positive ESI mode, 376 →123, 46 eV for haloperidol, 380 →123, 46 eV for haloperidol-d_4. The limit of quantification for haloperidol was 2.5 ng/ml.

2-1-H Brain and liver *ex vivo* dextrorphan formation

The probe substrate dextromethorphan undergoes CYP2D-specific *O*-demethylation to dextrorphan (Schmid et al., 1985). The rate of e*x vivo* dextrorphan formation from dextromethorphan hydrobromide (Sigma-Aldrich Canada, Oakville, Canada) was used to assess CYP2D enzyme activity. Cerebellum was used to assess CYP2D activity in brain due to high CYP2D expression in rodents (Cheng et al., 2013; Yue et al., 2008) and humans (Miksys et al., 2002). To assess dextrorphan formation by brain membranes, 1.5 mg cerebellar membrane protein, 40 µM dextromethorphan, and 1 mM NADPH were incubated in 1 ml artificial cerebrospinal fluid (ACSF) for 3 hours at 37°C under 5% CO_2 (Tyndale et al., 1999). Reactions were stopped with an equal volume of hexane-butanol (95:5 v/v).

The rat liver microsomal enzyme assay was adapted from a protocol used for mice (Siu et al., 2006). Briefly, rat livers were thawed on ice and homogenized in 1.15% KCl buffer, centrifuged at 9,000 g for 30 min. The supernatants were centrifuged at 100,000 g for 60 min and the resultant microsomal pellets were resuspended in a final volume of 1.15% KCl (µL) equal to wet weight (mg). To assess dextrorphan formation by liver microsomes, 0.5 mg microsomal protein, 2.5 µM dextromethorphan, 1 mM NADPH were incubated in 0.5 ml 100 mM potassium phosphate buffer for 5 minutes at 37°C (Kerry et al., 1993). Reactions were stopped with an equal volume of hexane-butanol (95:5 v/v)**.**

Samples from both brain membrane and liver microsome were extracted with an additional 5 ml hexane-butanol (Flores-Perez et al., 2004), with 50 ng 2-benzoxazolinone as internal standard, dried at 37°C under nitrogen, and the residue re-dissolved in mobile phase. Compounds were resolved at ambient temperature on an Agilent 1200 high performance liquid chromatography (HPLC) equipped with a ZORBAX Bonus-RP column (5 µm, 250×4.6 mm; Agilent

Technologies, Mississauga, Canada). 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 minutes 100-70% solvent A at 0.8 ml/min, 14-27 minutes 70-100% solvent A at 0.8-1.2 ml/min, 27-28 minutes 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 retention times of 22.6, 10.5, and 13.3 minutes for dextromethorphan, dextrorphan, and 2-benzoxazolinone, respectively, and a limit of quantification of 5 ng/ml for dextromethorphan and dextrorphan.

2-1-I Lipid peroxidation

The effect of inhibiting CYP2D in brain during chronic haloperidol exposure on brain and plasma lipid peroxidation was monitored by assessing malondialdehyde formed by lipid peroxidation using a fluorescent detection kit for thiobarbituric acid reactive substances according to the manufacturer's instructions (Caymen Chemical Company, Ann Arbor, MI).

2-1-J Data analyses

Data were analyzed with GraphPad Prism (version 6.05; La Jolla, California, USA) by independent samples t-tests, or by ANOVA followed by either planned comparisons using independent samples t-tests or post hoc testing adjusted for multiple comparisons (Tukey's). Relationships between data sets were assessed by Spearman's (catalepsy) and Pearson (vacuous chewing movements) correlation coefficients. All values are expressed as mean ± standard error of mean unless otherwise stated. A value of $p < 0.05$ was considered statistically significant.

2-2 Study 2: Propranolol is a mechanism-based inhibitor of CYP2D and CYP2D6 in humanized CYP2D6-transgenic mice: effects on activity and drug response

2-2-A Animals and housing conditions

Adult (8-12 weeks old) male TG (Cheng et al., 2013) and WT C57BL/6 mice (Charles River, St-Constant, Canada) were housed in groups of three to four under a 12-hour light/dark cycle with water and chow supplied *ad libitum*. Procedures were conducted in the light phase.

The derivation of these TG mice has previously been described (Cheng et al., 2013). Briefly, TG were produced by microinjecting fertilized FVB/N mouse eggs with the *CYP2D6* gene (Genbank accession number BX247885, PAC clone RP4-669P10). The insert contained the complete human *CYP2D6* gene sequence (exon 1-9), including the 5'- and 3'- flanking sequences, as well as the pseudogenes *CYP2D7P1* and *CYP2D8P1*. TG founders were mated with C57BL/6 mice (WT). After successive matings, confirmed homozygous TGs were established. Both polymerase chain reaction and Southern blot analyses were used to confirm the incorporation of the full length *CYP2D6* gene. Within our own breeding program, TG homozygosity was confirmed by crosses with WT. All TG mice used for the study were homozygous; all TG pups were genotyped for the presence of the human CYP2D6 transgene prior to use according to the published methods (Cheng et al., 2013).

2-2-B Liver *ex vivo* dextrorphan formation

Liver microsomes were prepared as previously described (Siu et al., 2006). Briefly, mouse livers were thawed on ice and homogenized in 1.15% KCl buffer, centrifuged at 9,000 g for 20 min,

followed by the supernatant being centrifuged at 100,000 g for 90 min. The resultant microsomal pellets were resuspended in a final volume (µL) of 1.15% KCl equal to half the liver wet weight (mg). Pooled (n=7) liver microsomes from untreated mice from each mouse line were used for baseline and inhibition assessments. Pooled human liver microsomes (Xenotech, Lenexa, USA) were used to compare *ex vivo* CYP2D metabolism between TG mice and humans. The assay conditions were adapted from Felmee et al. (2008), with time and protein concentration optimized for linear dextrorphan formation. Final incubation concentrations contained 100 mM potassium phosphate buffer (pH 7.4) and 1 mM NADPH. After preincubation with 50 µg microsomal protein (final concentration of 0.1 µg/µl) from TG, WT, or human liver for 2 minutes at 37°C, reactions were initiated by adding 50 µL of dextromethorphan (final concentration of 0.3-100 µM) for a total volume of 500 µL. Reactions were stopped with 500 µL of hexane-butanol (95:5 v/v) after 10 minutes.

Ex vivo dextrorphan formation from dextromethorphan by liver microsomes was quantified using HPLC, with standard curves for dextromethorphan and dextrorphan (5-500 ng/ml) as described in Section 2-1-H.

2-2-C Acute haloperidol and catalepsy

After a 5-minute acclimatization, the latency to remove a mouse's forepaws from a surface raised 4 cm above the cage floor was recorded, with a 420 second cut-off. To examine the dose-response relationship, sc injections of 0.05, 0.1, and 0.2 mg/kg haloperidol (n=3-7 per dose) were administered and catalepsy was assessed before (baseline, time 0) and at 15, 30, 45, 60, 90, 120, 150, and 180 minutes after injection.

2-2-D Plasma haloperidol levels

Saphenous vein blood samples were taken at 60 and 180 minutes after haloperidol injection to assess the effects of pre-treatments on plasma haloperidol levels. Blood was collected at equal times before and after the time when catalepsy was assessed to minimize the stress from blood collection on catalepsy testing while ensuring that the earliest blood draw was after haloperidol T_{max} in mice (Zetler and Baumann, 1985). The effects of *in vivo* 24-hour ip vehicle and propranolol pre-treatments were tested within animal with a two-week washout period between tests. Plasma samples were prepared and analyzed by LCMS as previously described in Section 2-1-G with some modifications. Briefly, 0.5 ng, rather than 12.5 ng, of the internal standard haloperidol-d4 was dissolved in 30:70 5 mM ammonium formate/acetonitrile. This was then added to each sample and to haloperidol calibration standards (1-500 ng/ml). No haloperidol metabolites were able to be measured.

2-2-E *In vitro* propranolol inhibition of *ex vivo* dextrorphan formation

A schematic of the preincubation schedule for inhibitory studies can be found in Figure 3.

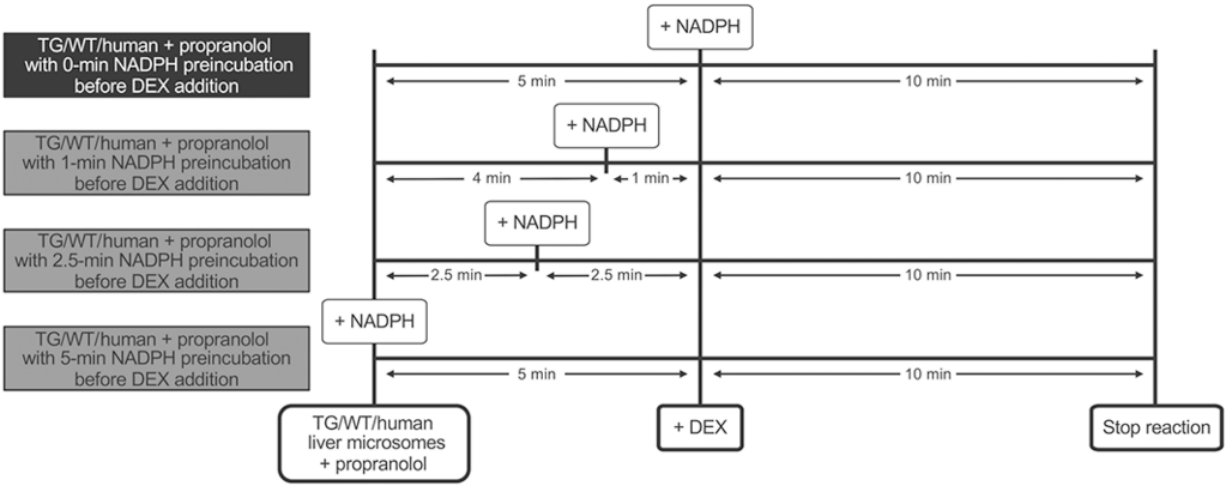


Figure 3. Schematic time courses of the conditions used for in vitro propranolol incubations with different preincubation times. *Pooled liver microsomes of TG, WT, and humans were co-incubated with propranolol, and NADPH was added at different times (listed above) before dextromethorphan (DEX) was added to each sample, and the reaction was carried out for another 10 minutes*

A Dixon plot (Dixon, 1953) is a graphical method of solving for a K_i value, an inhibition constant that denotes the dissociation equilibrium constant of the inhibitor-bound enzyme complex (Burlingham and Widlanski, 2003). To generate Dixon plots, reaction mixtures containing 50 µg microsomal protein (final concentration of 0.1 µg/µl) from TG or WT liver and propranolol (final concentration of 0-50 nM) in 100 mM potassium phosphate buffer were prewarmed for 2 minutes at 37°C before adding NADPH (final concentration of 1 mM). After a 5-minute preincubation, reactions were initiated by adding dextromethorphan (final concentration of 2.5, 5, or 10 µM) for a total volume of 500 µL.

An IC_{50} refers to an inhibitor concentration required to halve the enzymatic reaction rate (under specific conditions) (Burlingham and Widlanski, 2003). To generate IC_{50} plots, reaction mixtures containing 50 µg microsomal protein (final concentration of 0.1 µg/µl) from TG, WT, or human liver and propranolol (final concentration of 0-100 µM) were prewarmed for 2 minutes at 37°C before adding NADPH after either 0 or 5 minutes during a subsequent 5 minute preincubation (Figure 3). Reactions were initiated by adding dextromethorphan (final concentration of 5 µM, approximate K_m) for a total volume of 500 µL.

To generate inactivation curves, reaction mixtures containing 50-µg microsomal protein (final concentration of 0.1 µg/µl) from TG, WT, or human liver and propranolol (final concentration of 0-10 µM) were prewarmed at 37°C for 2 minutes, before adding NADPH after 0, 2.5, 4, or 5 minutes during a subsequent 5 minute preincubation (Figure 3).

Ex vivo dextrorphan formation from dextromethorphan by liver microsomes was quantified using HPLC, with standard curves for dextromethorphan and dextrorphan (5-500 ng/ml) as described in Section 2-1-H.

2-2-F Effect of inhibiting CYP2D by *in vivo* 24-hour pre-treatment with ip propranolol

Mice were pre-treated *in vivo* with either ip propranolol (20 mg/kg in saline) or ip saline (n=10 per group) 24 hours before experimental testing. After 24 hr, ip injections of dextromethorphan (30 mg/kg in saline) were administered and saphenous vein blood was collected 30 minutes later. Blood was collected after dextromethorphan T_{max} (estimated as 15 minutes in mice) (Sakai et al., 2014) so that we are at the descending limb of the dextromethorphan curve. Mice were immediately killed, and tissues were collected and stored at -80°C. Dextromethorphan and dextrorphan levels in plasma were quantified using HPLC, with standard curves for

dextromethorphan and dextrorphan (5-500 ng/ml) as described in Section 2-1-F. The ratio of plasma dextrorphan/dextromethorphan was used as an index of *in vivo* hepatic CYP2D activity. Given that the half-life of propranolol in mice is ~45 minutes (Levy et al., 1976), the 24-hour pre-treatment represents approximately 32 half-lives and thus we did not attempt to measure propranolol concentrations.

Liver microsomes were derived from *in vivo* drug pre-treated TG and WT as described in Section 2-2-B. *Ex vivo* liver microsome incubations were performed at a later date using liver microsomes stored at -80°C and the rate of *ex vivo* dextrorphan formation from dextromethorphan was quantified by HPLC as described in Section 2-1-F.

To test how inhibition of peripheral CYP2D affects catalepsy, mice received either ip propranolol or ip saline (n=10 per group) 24 hours prior to 0.1 mg/kg sc haloperidol and catalepsy was measured 120 minutes later.

2-2-G Data analyses

The data was analyzed using GraphPad Prism (version 6.0c; La Jolla, California, USA). Michaelis-Menten parameters, K_m and V_{max}, as well as the corresponding standard error for both parameters were estimated using non-linear regression. Michaelis-Menten parameters were also confirmed using the Eadie-Hofstee method. Intrinsic clearance (CL_{int}) was calculated as the ratio of V_{max} to K_m. The inhibition constant K_i was determined according to Dixon (Dixon, 1953). IC_{50} was estimated using non-linear regression and calculated as the concentration of propranolol that was halfway between the top and the bottom plateaus of the curve for a one-site enzyme (Neubig et al., 2003). For two-enzyme systems, $IC_{50,High}$ and $IC_{50,Low}$ are defined as IC_{50} for the high affinity sites and low affinity sites, respectively. To estimate inactivation constants, the initial rate constant of

inactivation of dextrorphan formation by each propranolol concentration (K_{obs}) was assessed by linear regression analysis of the natural logarithm of the percentage of activity remaining versus preincubation time data (Kitz and Wilson, 1962). Thereafter, the K_{obs} values were used to determine the inhibitor concentration needed to cause a half-maximal rate of enzyme inactivation (K_I) and the maximal rate of inactivation ($k_{inactivation}$). The K_I and $k_{inactivation}$ were estimated by non-linear regression using the following equation (Jones et al., 1999): $K_{obs} = \frac{k_{inactivation}*[I]}{K_I+[I]}$. Plasma dextrorphan/dextromethorphan ratios and hepatic dextrorphan formation velocities were analyzed by two-tailed, unpaired sample t-tests, while catalepsy scores and plasma haloperidol levels were analyzed by two-tailed, paired samples t-tests. Statistical analyses were only performed with measures that had at least five animals per group; n=10 individual animals per group (treatment and mouse line) were the basis of the independent values, using within or between statistical analyses as indicated. All values are expressed as mean ± standard error of mean unless otherwise stated. A value of $p < 0.05$ was considered statistically significant. Data was normalized to values relative to mean saline pre-treatment controls within each mouse line to illustrate the effect of *in vivo* 24-hour ip propranolol pre-treatment; however, all statistical analyses were run with raw, non-normalized data. Outliers were included in data analysis and figures.

2-3 Study 3: Human CYP2D6 is functional in brain *in vivo*: evidence from humanized mice

2-3-A Animals and housing conditions

Adult (8-12 weeks old) male TG (Cheng et al., 2013) and WT C57BL/6 mice (Charles River, St-Constant, Canada) were housed in groups of three to four under a 12 hr light/ 12 hr dark cycle with water and chow supplied *ad libitum*. Procedures were all conducted in the light phase. The homozygosity of TG mice was confirmed as described in Section 2-2-A.

2-3-B Mouse icv cannulation surgery

Mice were anesthetized with isoflurane (4% induction, 1-2% maintenance) and positioned in a stereotaxic frame. Stainless steel guide cannulas (28 ga.) were implanted into the right lateral cerebral ventricle for icv injections, with bregma coordinates dorsal-ventral 2.3 mm, lateral-medial -1.0 mm, and anterior-posterior -0.9 mm (Paxinos and Franklin, 2007). Mice were given the analgesic meloxicam (2 mg/kg sc) intraoperatively and daily for 3 days after surgery. They were allowed to recover for at least one week prior to testing. Cannula patency was maintained by sterile dummy cannulas, removed only for drug administration.

2-3-C Brain *ex vivo* dextrorphan formation

Assay conditions were adapted from the rat brain membrane protocol as described in Section 2-1-F. Optimal brain membrane concentration and incubation times were identified for linear dextrorphan formation. Brain cerebellar membranes were prepared as previously described (Siu et al., 2006; Tyndale et al., 1999). Briefly, whole individual cerebella (approximately 300-400 µg) were thawed on ice and homogenized in ACSF, centrifuged at 1,700 g for 5 min, followed by the

supernatant being centrifuged at 100,000 g for 90 min. The resulting membrane pellets were resuspended in 150 µL volume of ACSF. Freshly prepared membranes from whole individual cerebella were incubated with 50 µM dextromethorphan (approximate concentration at V_{max} derived from Michaelis-Menten plots of TG liver microsomes incubated between 0.3-100 µM dextromethorphan; see results reported in Section 3-2-A) and 1 mM NADPH in ACSF (pH 7.4) for 90 minutes at 37°C under 95% O_2/5% CO_2 in a final volume of 1 ml. Dextrorphan tartate (5 ng dextrorphan base, Sigma-Aldrich Canada, Oakville, Canada) was added to each stopped incubate immediately before extraction to ensure that dextrorphan levels were above the limit of detection. The dextrorphan measured in each sample then had the spiked dextrorphan amount subsequently subtracted to calculate the amount of enzymatically formed dextrorphan. Reactions were stopped with an equal volume of hexane-butanol (95:5 v/v). A variety of incubation controls (e.g. buffer + NADPH, buffer + NADPH + dextromethorphan, buffer + NADPH + fresh membranes/microsomes, buffer + NADPH + dextromethorphan + denatured membranes/microsomes) were tested and no dextrorphan formation was detected in any of these controls, thus confirming that the dextrorphan identified from brain and liver incubations was enzymatically formed. Brain samples were extracted and analyzed as described in Section 2-1-H.

2-3-D Effect of inhibiting CYP2D by *in vivo* 4-hour pre-treatment with icv propranolol

TG and WT were randomly assigned to icv pre-treatment with 2 µL of either 40 µg/µl propranolol (80 µg propranolol; ~2.67 mg/kg for an average 30 g mouse) in vehicle (sterile 20% w/v 2-hydroxypropyl-β-cyclodextrin) or vehicle alone. After an *in vivo* 4-hour pre-treatment with icv propranolol or vehicle (n=12 per group), an ip injection of 30 mg/kg dextromethorphan in sterile saline was administered. The ratio of dextrorphan/dextromethorphan levels in brain and plasma was used as an index of *in vivo* CYP2D activity. A saphenous vein blood sample was collected 30

minutes after the dextromethorphan injection and mice were then immediately sacrificed and brains and livers were dissected. For brain drug levels, a half-brain minus the cerebellum was homogenized in 0.25 M Na_2CO_3 (1:5 w/v), centrifuged at 12,500 g for 20 min, and the supernatant was assayed. For plasma drug levels, blood samples were centrifuged at 5,000 g for 10 minutes and plasma was collected and assayed. To assess *ex vivo* CYP2D activity in the brain after the 4-hour *in vivo* icv drug pre-treatment, e*x vivo* brain membrane incubations were performed the same day as sacrifice to prevent loss of CYP2D activity from the freezing of brain tissues (Tyndale et al., 1999). Cerebellar membranes were made and tested for activity as described in Section 2-3-C. To assess *ex vivo* CYP2D activity in the liver after the 4-hour *in vivo* icv drug pre-treatment e*x vivo* liver microsome incubations were performed at a later date using liver microsomes stored at -80°C. TG and WT liver microsomes were made and their *ex vivo* dextrorphan formation were measured as described in Section 2-2-B.

Dextromethorphan and dextrorphan levels *in vivo* in brain and in plasma, and *ex vivo* by brain membranes and by liver microsomes were quantified using HPLC. Standard curves for dextromethorphan and dextrorphan (5-500 ng/ml) are the same as described in Section 2-1-H.

2-3-E Effect of inhibiting CYP2D by *in vivo* 24-hour pre-treatment with icv propranolol

TG and WT were randomly assigned to an *in vivo* 24-hour icv pre-treatment with either propranolol or vehicle, as described in Section 2-3-D. After an *in vivo* 24-hour pre-treatment with icv propranolol or vehicle (n=11-12 per group), an ip injection of dextromethorphan was administered, as described in Section 2-3-D. A saphenous vein blood sample was collected 30 minutes after the dextromethorphan injection and mice were then immediately sacrificed and brains and livers were dissected. *In vivo* brain and liver drug levels, and *ex vivo* CYP2D activity in the brain and liver were measured, as described in Section 2-3-D.

Catalepsy in mice was measured as described in Section 2-2-C. To test the impact of inhibiting CYP2D6 in brain on catalepsy response, TG and WT (n=10 per group) were given an *in vivo* 24-hour pre-treatment with icv propranolol or vehicle prior to a 0.1 mg/kg sc haloperidol injection and catalepsy was measured 120 minutes post-haloperidol. Mice acted as their own controls, given a pre-treatment of either *in vivo* 24-hour icv propranolol or vehicle, and after a two-week washout, given the alternative pre-treatment. For both mouse lines, the order of pre-treatment had no effect on catalepsy (all $p > 0.2$). Timing and dose were selected from the dose response experiment described in Section 2-2-C and results reported in Section 3-3-D.

To confirm that the *in vivo* 24-hour icv drug pre-treatments had no effect on plasma haloperidol levels, saphenous vein blood samples were collected and analyzed as previously described in Section 2-2-D.

2-3-F Data analyses

The data were analyzed using GraphPad Prism (version 6.0c, La Jolla, California, USA). Mouse line comparisons of brain dextrorphan/dextromethorphan ratio and brain dextrorphan formation were analyzed by two-tailed, unpaired t-tests. It was expected that *in vivo* icv propranolol pre-treatment (both 4- and 24-hour) would result in inhibition of CYP2D in mouse brain based on previous similar inhibition work in rats (McMillan et al., 2019; McMillan and Tyndale, 2015). Thus, analyses of the results assessing the impact of *in vivo* icv propranolol pre-treatment (both 4- and 24-hour) was preplanned as within mouse line analyses. Pre-treatment comparisons of brain dextrorphan/dextromethorphan ratio, brain dextrorphan formation rate, plasma dextrorphan/dextromethorphan ratio, and liver dextrorphan formation rate were analyzed between animal and within mouse line by two-tailed, unpaired t-tests. Catalepsy scores from the dose response were first analyzed by two-way repeated measures ANOVA separately for each mouse

line (dose x time). This was followed by a two-way ANOVA of catalepsy data at 120 minutes post-haloperidol injection (dose x mouse line). Post-hoc Bonferroni analyses were then performed. Pre-treatment comparisons of catalepsy data at 120 minutes post-haloperidol injection and plasma haloperidol levels were each analyzed within animal (two-week crossover of pre-treatment) and within mouse line by two-tailed, paired t-tests. All values are expressed as mean ± standard deviation unless otherwise stated. A value of $p < 0.05$ was considered statistically significant. Data were provided in the figures as normalized to mean vehicle pre-treatment controls within each mouse line to illustrate the relative impact of 4- and 24-hour pre-treatment with icv propranolol; all statistical analyses were performed with raw, non-normalized data.

3 Results

3-1 Study 1: CYP2D enzymatic metabolism in the rat brain alters acute and chronic haloperidol side-effects by different mechanisms

The role of CYP2D in rat brain has been demonstrated *in vivo* with the CYP2D substrates codeine (McMillan and Tyndale, 2015) and oxycodone (McMillan et al., 2019), where the selective inhibition and induction of CYP2D in rat brain resulted in behavioural changes without changes in plasma drug levels. In order to improve our understanding of the role that manipulating CYP2D in brain plays on central drug metabolism and response, we extended this to other, non-opioid, centrally-acting CYP2D substrates. Therefore, we studied the effect of manipulating CYP2D in brain on the metabolism of, and behaviour from, haloperidol an antipsychotic with an acute and chronic side effect profile that models side effects in humans from short- and long-term antipsychotic use. Haloperidol-induced behaviours from acute (catalepsy) and chronic (vacuous chewing movements) treatments were measured in rats with inhibition and/or induction of CYP2D in brain. Results have been published (doi: 10.1016/j.pnpbp.2017.04.030).

3-1-A Acute haloperidol-induced catalepsy is dose-dependent

Catalepsy reached a plateau by about 30 minutes post-haloperidol injection at most doses and remained stable through to final testing at 110 minutes (Figure 4a). Catalepsy was dose-dependent with 0.125 mg/kg sc haloperidol in the mid-range of the dose-response curve (0.03-0.5 mg/kg), as illustrated at 60 minutes post injection (Figures 4a and 4b).

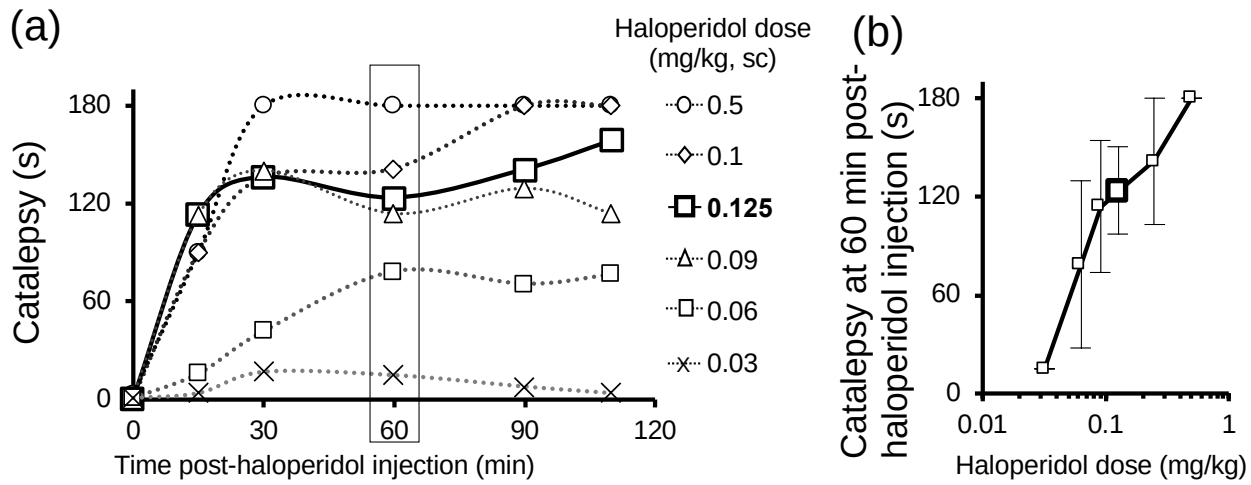


Figure 4. Acute haloperidol-induced catalepsy is dose dependent. (a) *Catalepsy was haloperidol dose-dependent, reached a maximum by 30-60 minutes at all doses, and persisted throughout testing (n=2-5/dose).* ***(b)*** *At 60 minutes post-haloperidol (boxed in a), 0.125 mg/kg sc was at the mid-point of the dose-response curve (R^2=0.69, p=0.04). Data are expressed as mean ± standard error of mean.*

Brain and plasma haloperidol levels decreased over time (Figures 5a and 5b). Average haloperidol levels, derived from area under the catalepsy-time curve ($AUC_{0\text{-}110}$), were 42 ng/g in brain and 8.6 ng/ml in plasma.

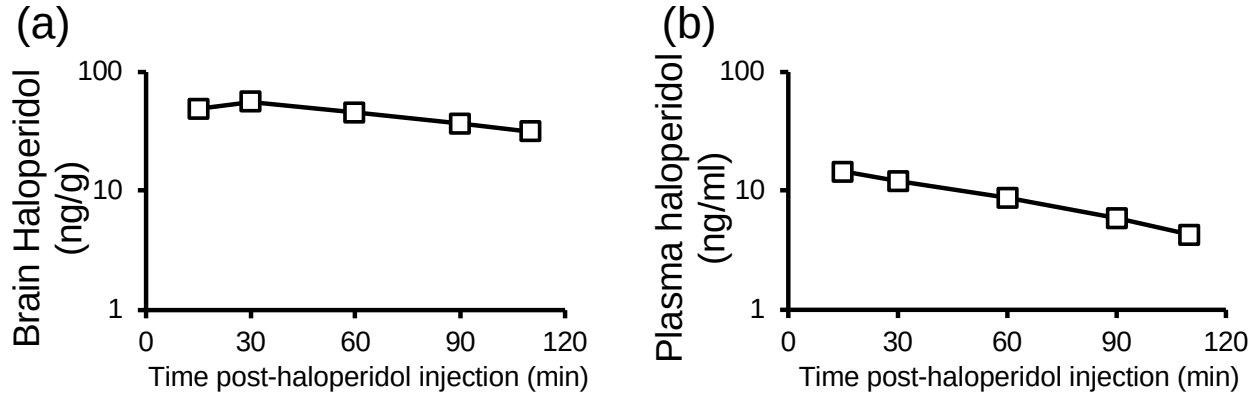


Figure 5. Brain and plasma haloperidol levels after an acute haloperidol injection. (a) *Brain and* ***(b)*** *plasma haloperidol levels declined over 110 minutes post-haloperidol (0.125 mg/kg sc) in rats (n=4/time). Data are expressed as mean ± standard error of mean.*

3-1-B Acute haloperidol-induced catalepsy was reduced by inhibition and increased by induction of CYP2D in brain

Pre-treatment with *in vivo* icv propranolol, 24 hours prior to testing, significantly reduced catalepsy at 30 (p=0.03), 60 (p=0.02), and 90 (p=0.001) minutes after 0.125 mg/kg sc haloperidol (Figure 6a; repeated measures ANOVA, F(1, 20) = 6.047, p=0.02), resulting in a lower $AUC_{0\text{-}90}$ (t=-2.112, df=22, p=0.04) compared to icv vehicle. At 60 minutes, catalepsy following inhibitor pre-treatment corresponded to catalepsy observed in the absence of inhibitor at an estimated dose of 0.08 mg/kg haloperidol (Figure 4b), indicating a rightward shift in the dose-response curve. In a separate experiment, 7-day pre-treatment with sc nicotine, an inducer of CYP2D in brain, significantly increased catalepsy at 60 (p=0.03) and 90 (p=0.03) minutes after haloperidol injection (Figure 6b; repeated measures ANOVA, F(1, 43) = 3.127, p=0.04), resulting in a higher

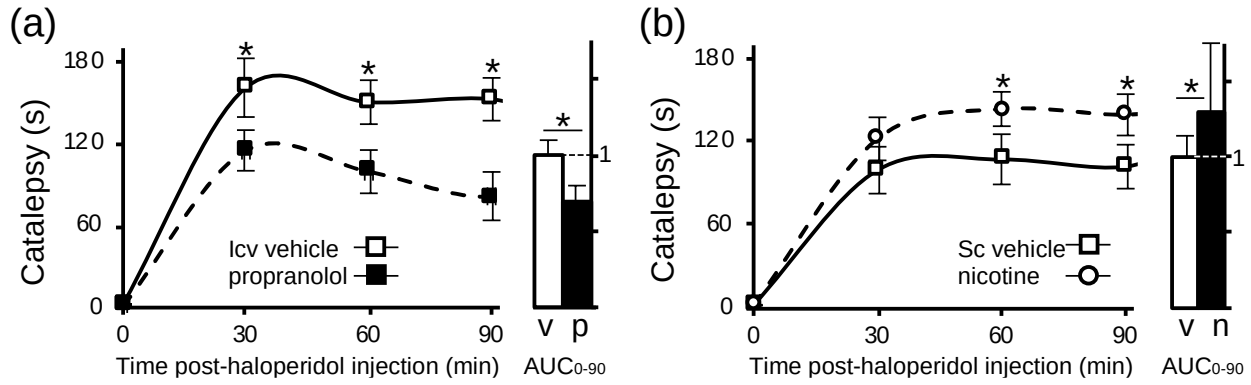


Figure 6. Acute haloperidol-induced catalepsy is modulated by CYP2D activity in brain. (a) *Catalepsy was decreased with inhibition of CYP2D in brain by in vivo 24-hour pre-treatment with icv propranolol (n=12) at 30, 60, and 90 minutes post-haloperidol (0.125 mg/kg sc), and the area under the catalepsy-time curve ($AUC_{0\text{-}90}$) was lower compared to controls given in vivo 24-hour pre-treatment with icv vehicle (n=14).* ***(b)*** *Catalepsy was increased with induction of CYP2D in brain by 7-day sc nicotine pre-treatment (n=23) at 60 and 90 minutes post haloperidol (8 hours after the seventh nicotine injection), and the area under the catalepsy-time curve was higher compared to controls given 7-day sc vehicle pre-treatment (n=22). Baseline scores (after icv propranolol or sc nicotine but before haloperidol injection) are shown at time 0 in (a) and (b) and indicate no catalepsy due to pre-treatments. Data are expressed as mean ± standard error of mean. *p < 0.05 calculated using repeated measures ANOVA on the raw data.*

$AUC_{0\text{-}90}$ (t=-1.762, df=43, p=0.04) compared to sc vehicle. At 60 minutes, catalepsy following nicotine pre-treatment corresponded to catalepsy observed in the absence of inducer at an estimated dose of 0.17 mg/kg (Figure 4b), indicating a leftward shift in the dose-response curve. Baseline data, indicated by time 0 in Figures 6a and 6b, showed no catalepsy after either 24-hour icv propranolol or 8-hour sc nicotine pre-treatments before haloperidol injection.

3-1-C Acute haloperidol-induced catalepsy was increased by induction of CYP2D in brain, and this was reversed by subsequent inhibition

To extend these findings, a four-arm pre-treatment experiment assessed catalepsy at 60 minutes

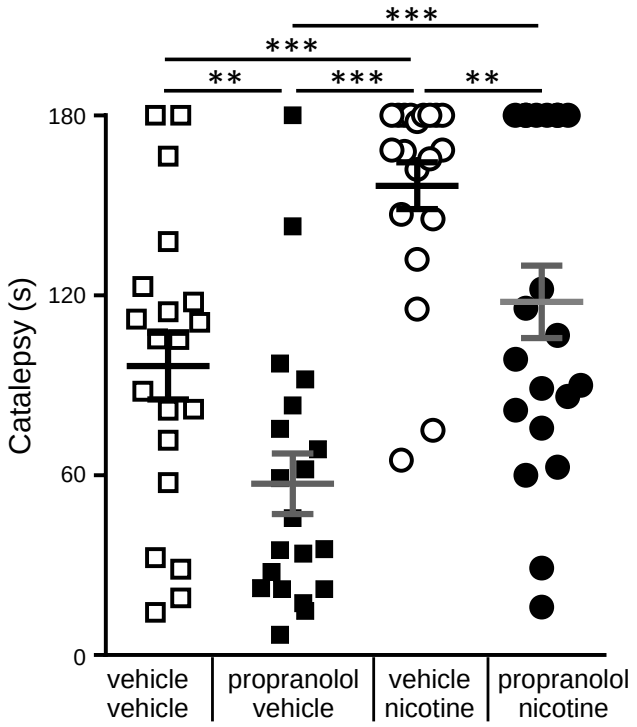


Figure 7. Acute haloperidol-induced catalepsy is decreased by in vivo 24-hour pre-treatment with icv propranolol and increased by treatment with 7-day sc nicotine. *At 60 minutes post-haloperidol (0.125 mg/kg sc), catalepsy duration was decreased after in vivo icv propranolol pre-treatment inhibition of CYP2D in brain, was increased after nicotine pre-treatment induction of CYP2D in brain, and was similar to vehicle pre-treatment control levels after propranolol inhibition of nicotine induced CYP2D in brain (n=20/group). Data are expressed as mean ± standard error of mean. **p<0.01, ***p<0.001 calculated using two-way ANOVA, post-hoc test for multiple comparisons on the raw data.*

after 0.125 mg/kg haloperidol. Catalepsy was again reduced following pre-treatment with *in vivo* 24-hour icv inhibitor propranolol, and increased following pre-treatment with sc inducer nicotine; this increase in catalepsy after induction of CYP2D in brain was reversed by icv inhibitor (Figure 7; inhibition, $F(1, 77) = 13.95$, $p<0.001$, induction $F(1, 77) = 33.38$, $p<0.001$, no interaction, $F(1, 77) = 0.0007$, $p=0.9$).

The assessment of *ex vivo* brain dextromethorphan metabolism confirmed that *in vivo* 24-hour icv propranolol inhibited and sc nicotine induced CYP2D activity in brain, and that icv propranolol inhibited the induced activity (Figure 8a; inhibition $F(1, 70) = 20.17$, $p<0.001$,

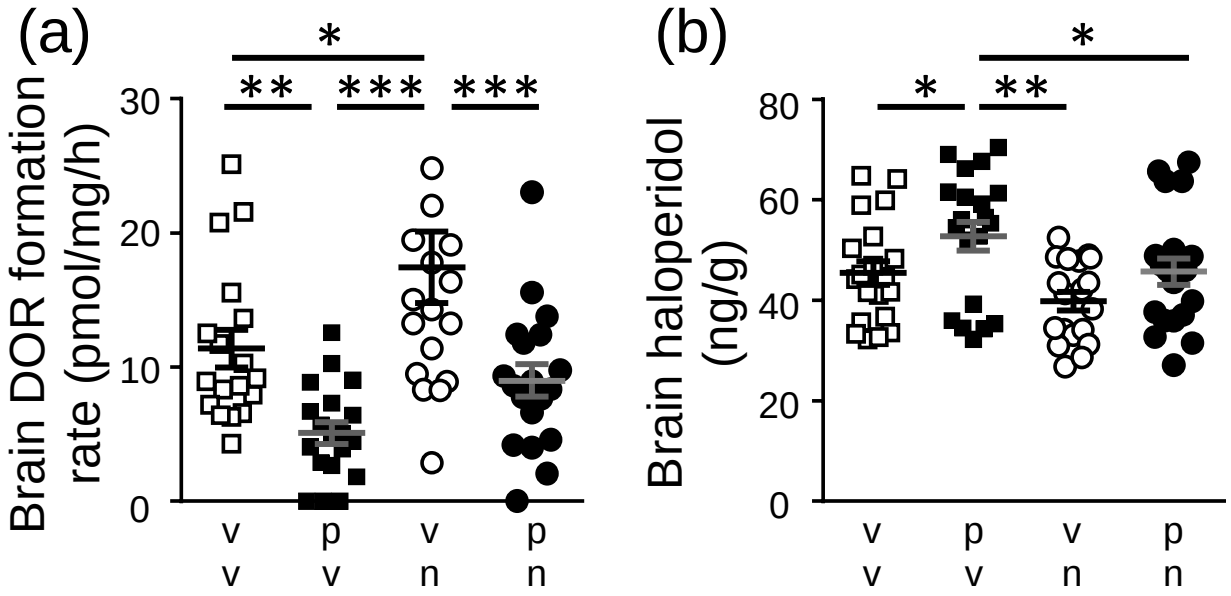


Figure 8. CYP2D activity in the brain is decreased by in vivo 24-hour pre-treatment with icv propranolol and increased by treatment with 7-day sc nicotine. (a) *Ex vivo CYP2D activity, as measured by the rate of dextrorphan (DOR) formation from dextromethorphan, in brain was decreased by inhibition, increased by induction, and was at control levels with inhibition of induced CYP2D in brain (n=20/group).* ***(b)*** *Consistent with CYP2D activity in brain, brain haloperidol levels were increased by inhibition, decreased by induction, and were at control levels with inhibition of induced CYP2D in brain. V- vehicle, p- propranolol, n- nicotine pre-treatments. Data are expressed as mean ± standard error of mean. *p<0.05, **p<0.01, ***p<0.001 calculated using two-way ANOVA, post-hoc test for multiple comparisons on the raw data.*

induction F(1, 70) = 9.23, p=0.003, no interaction F(1, 70) = 0.42, p=0.5). Consistent with these effects on CYP2D activity in brain, brain haloperidol levels were higher after inhibition, lower after induction, and similar to vehicle controls after inhibition of induced CYP2D in brain (Figure 8b; inhibition F(1, 75) = 7.17, p=0.009, induction F(1, 75) = 6.56, p=0.01, no interaction F(1, 75) = 0.08, p=0.8).

Catalepsy significantly correlated with CYP2D activity in brain (Figure 9, r=0.51, p<0.001).

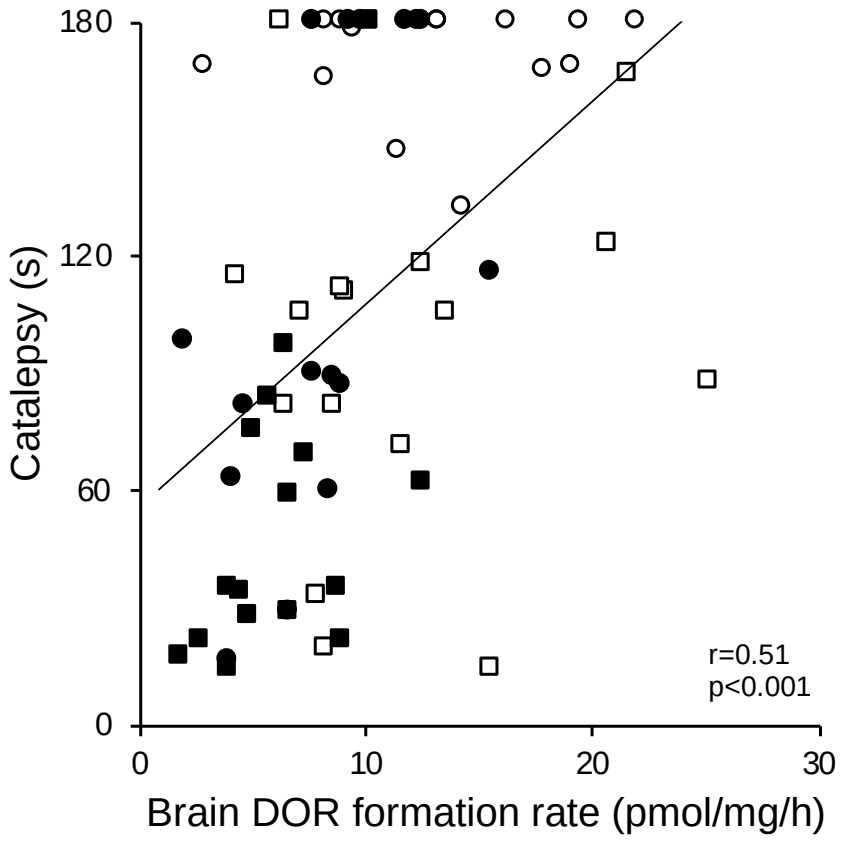


Figure 9. Acute haloperidol-induced catalepsy correlated with CYP2D activity in brain. *Catalepsy duration 60 minutes post-haloperidol (0.125 mg/kg sc) correlated positively with ex vivo CYP2D enzyme activity in brain, r=0.51, p<0.001. Rat pre-treatment assignments are as follows: hollow squares are vehicle-vehicle, filled squares are propranolol-vehicle, hollow circles are vehicle-nicotine, and filled circles are propranolol-nicotine.*

In contrast, neither *ex vivo* CYP2D activity in liver (Figure 10a) nor plasma haloperidol levels (Figure 10b) were significantly affected by inhibitor or inducer pre-treatments.

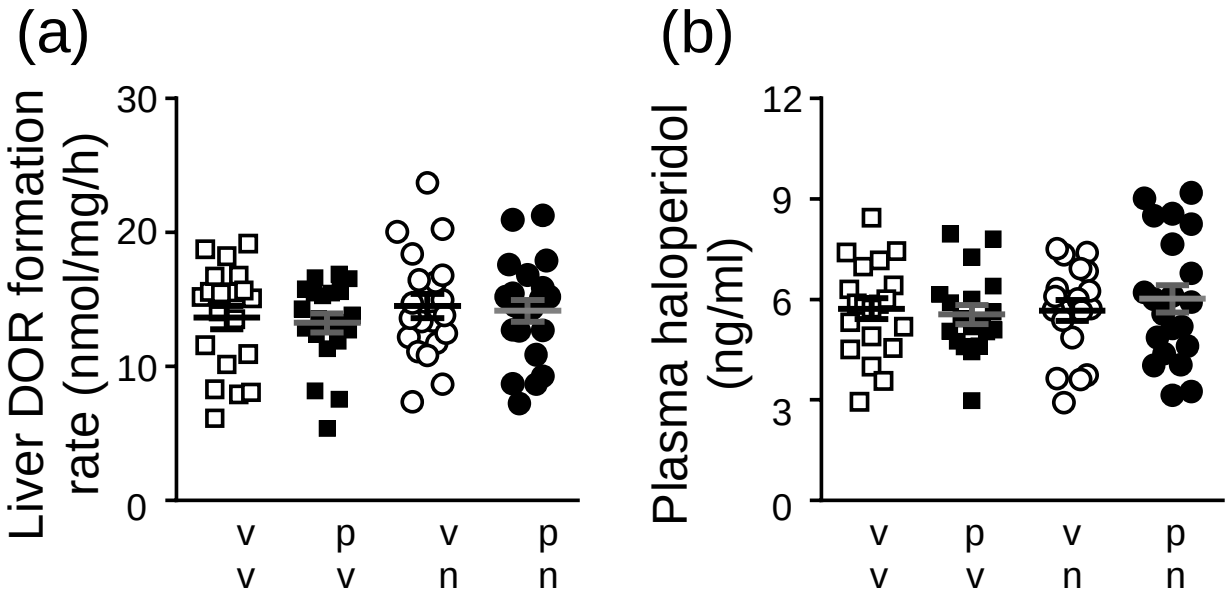


Figure 10. CYP2D activity in the liver is unchanged by in vivo 24-hour pre-treatment with icv propranolol and by treatment with 7-day sc nicotine. (a) *Ex vivo CYP2D activity in liver and* ***(b)*** *plasma haloperidol levels were unchanged by propranolol inhibition or nicotine induction of CYP2D in brain. v- vehicle, p- propranolol, n- nicotine pre-treatments. Data are expressed as mean ± standard error of mean. *p<0.05, **p<0.01, ***p<0.001 calculated using two-way ANOVA, post-hoc test for multiple comparisons on the raw data.*

Catalepsy did not correlate with CYP2D activity in liver (Figure 11, r=0.005, p=0.9).

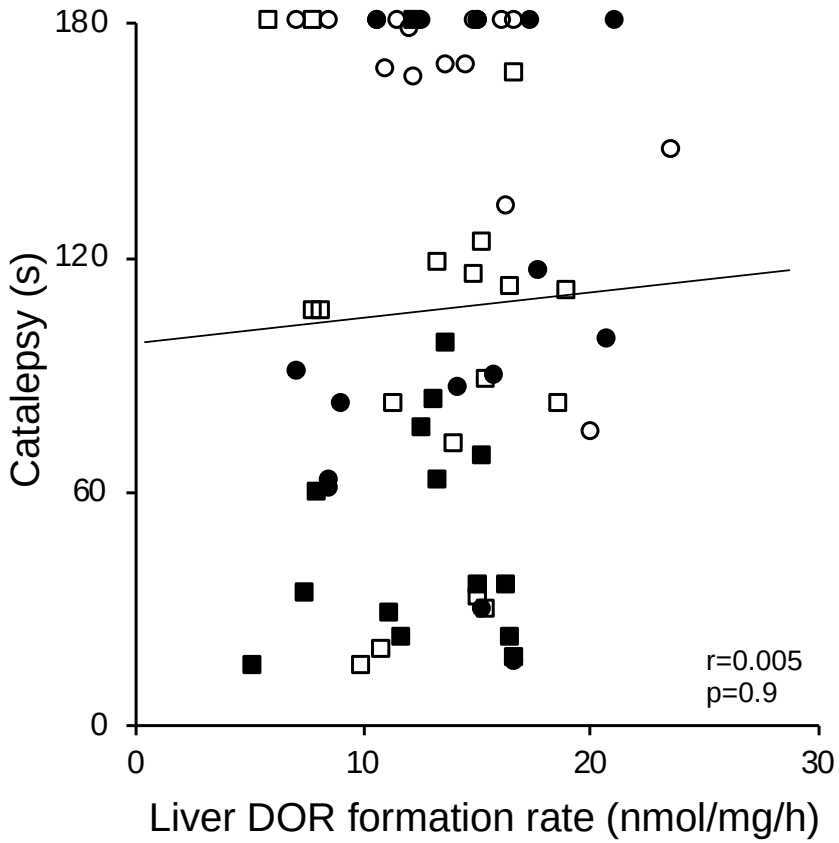


Figure 11. Acute haloperidol-induced catalepsy did not correlate with CYP2D activity in brain. *Catalepsy duration 60 minutes post-haloperidol did not correlate with ex vivo CYP2D enzyme activity in liver, r=0.005, p=0.9. Rat pre-treatment assignments are as follows: hollow squares are vehicle-vehicle, filled squares are propranolol-vehicle, hollow circles are vehicle-nicotine, and filled circles are propranolol-nicotine.*

This confirmed that neither *in vivo* 24-hour icv-delivered propranolol nor sc nicotine pre-treatments affected CYP2D metabolism in liver and resulting plasma haloperidol levels. Together, these data showing a decrease in catalepsy following inhibitor pre-treatment, and an increase in catalepsy following inducer pre-treatment, suggest that CYP2D-mediated metabolism in brain is sufficient to meaningfully alter brain drug and metabolite levels, and that a CYP2D-mediated haloperidol metabolite formed in brain may be responsible for catalepsy.

3-1-D Chronic haloperidol-induced vacuous chewing movements were increased by inhibition of CYP2D in the brain

The average vacuous chewing movements, during the last four weeks of haloperidol treatment, were significantly higher with *in vivo* 24-hour icv propranolol (versus vehicle), in both the first study (Figure 12a) and the larger replication study (Figure 12b). Baseline vacuous chewing movements (after *in vivo* 24-hour icv propranolol but before first haloperidol injection) are indicated at time 0 in Figure 12a and 12b. In Study 1 (Figure 12a) these were 0.2 ± 0.1 (icv vehicle) and 0.2 ± 0.1 (*in vivo* 24-hour icv propranolol); in Study 2 (Figure 12b) these were 2.4 ± 1.0 (icv vehicle) and 3.8 ± 3.8 (*in vivo* 24-hour icv propranolol).

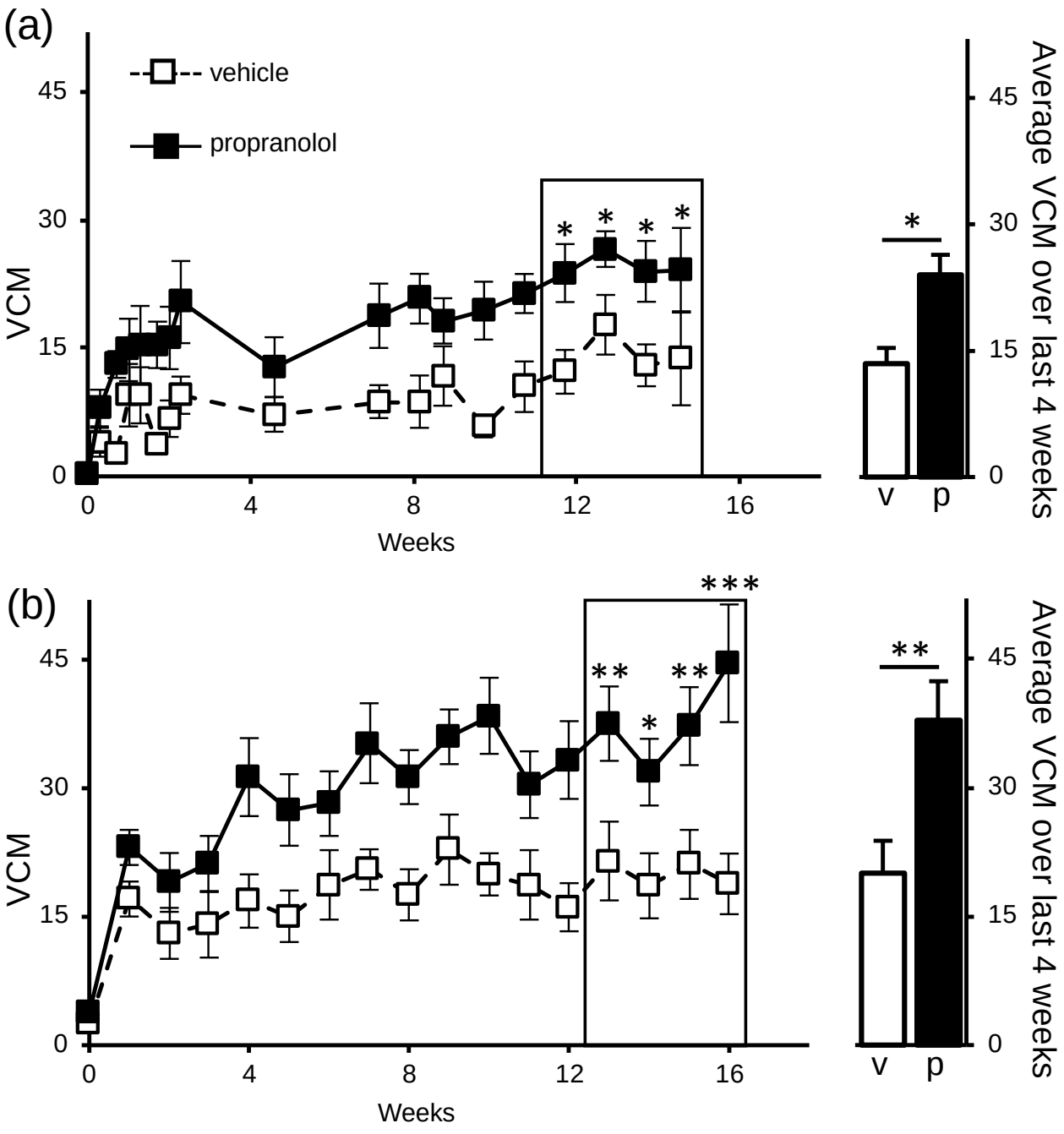


Figure 12. Chronic haloperidol-induced vacuous chewing movements (VCMs) are increased by inhibition of CYP2D in brain. *In replicate studies (**(a)**: pilot, n=4-5/group, **(b)**: full study, n=9-12/group), the average vacuous chewing movements in the last 4 weeks (boxed) were higher after in vivo 24-hour pre-treatment with icv propranolol compared to in vivo 24-hour pre-treatment with icv vehicle. v- vehicle, p- propranolol icv pre-treatments. Data are expressed as mean ± standard error of mean. *p<0.05, **p<0.01, ***p<0.001 calculated using post-hoc test for multiple comparisons for vacuous chewing movements, independent samples t-tests for 4-week average vacuous chewing movements and activity.*

Plasma haloperidol levels remained essentially constant both during the 21 days following im-haloperidol decanoate (Figure 13a, average levels of 11.8 ± 1.6 ng/ml for icv vehicle and 12.6 ± 2.6 for *in vivo* 24-hour icv propranolol, data from the first study) and also over the duration of the study (Figure 13b, average levels of 13.3 ± 2.5 ng/ml for icv vehicle and 11.8 ± 2.8 ng/ml for *in vivo* 24-hour icv propranolol, data from the second study).

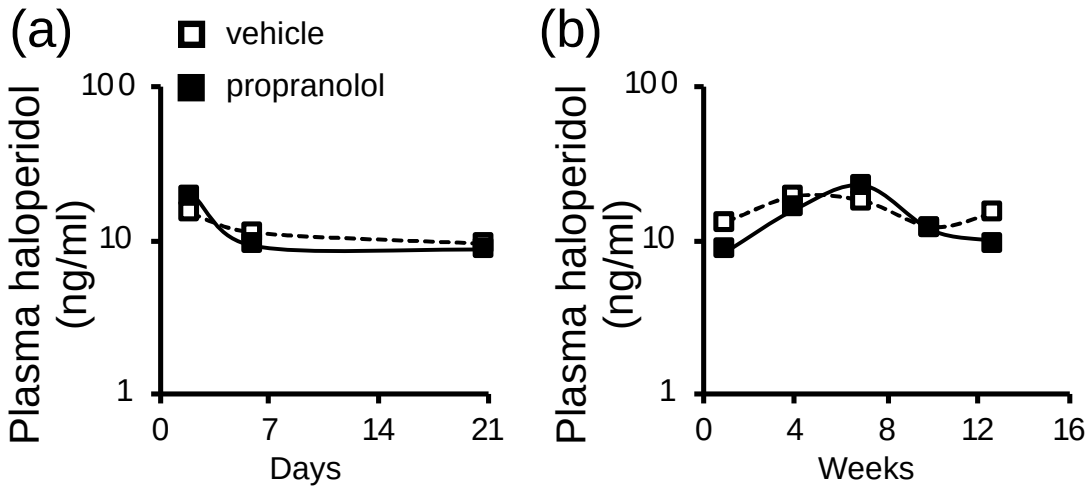


Figure 13. In vivo 24-hour pre-treatment with icv propranolol did not affect haloperidol levels in rat plasma. *Plasma haloperidol levels remained constant over* ***(a)*** *21 days after im haloperidol decanoate, and* ***(b)*** *over the 4 months of the study, with no difference between in vivo 24-hour pre-treatment with icv propranolol or vehicle.*

Assessment of *ex vivo* dextrorphan formation in brain confirmed that *in vivo* 24-hour pre-treatment with icv propranolol irreversibly inhibited CYP2D activity in brain (Figure 14a), consistent with higher brain haloperidol levels (Figure 14b).

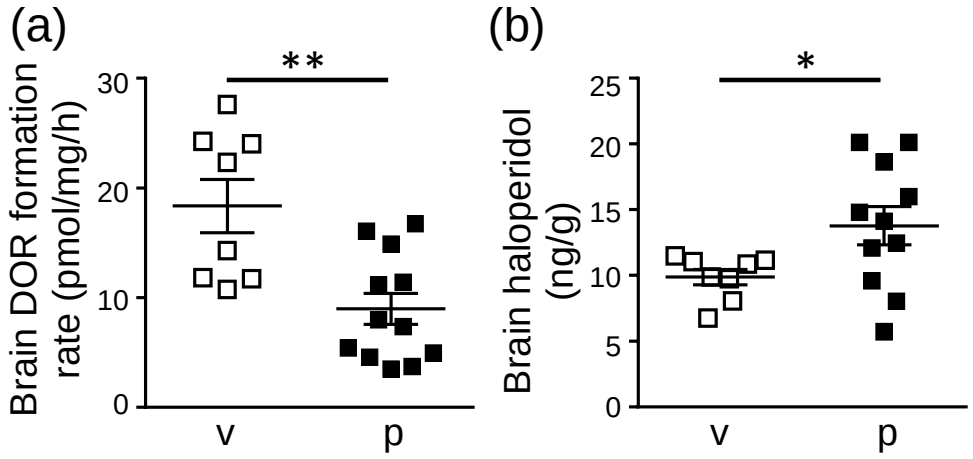


Figure 14. In vivo 24-hour pre-treatment with icv propranolol reduced ex vivo dextrorphan (DOR) formation without affecting haloperidol levels in rat brain. (a) *Ex vivo CYP2D activity in brain, as measured by the rate of dextrorphan formation from dextromethorphan, and* ***(b)*** *brain haloperidol levels were increased by icv propranolol pre-treatment. v- vehicle, p- propranolol icv pre-treatments. Data are expressed as mean ± standard error of mean. *p<0.05, **p<0.01 calculated using independent samples t-tests for activity and haloperidol levels.*

In vivo 24-hour pre-treatment with icv propranolol had no effect on *ex vivo* CYP2D activity in liver, consistent with a lack of effect on plasma haloperidol levels (Figures 15a and 15b).

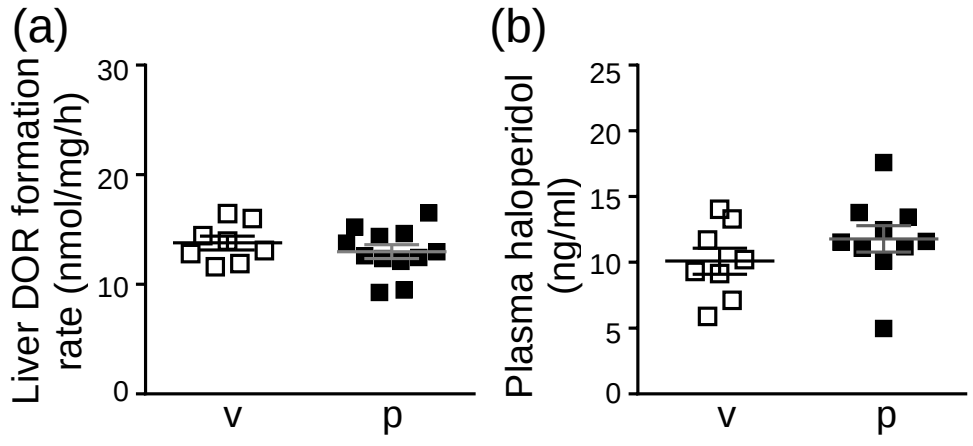


Figure 15. In vivo 24-hour pre-treatment with icv propranolol did not affect ex vivo dextrorphan (DOR) formation and haloperidol levels in rat liver and plasma. (a) *Ex vivo CYP2D activity in the liver as measured by the rate of dextrorphan formation from dextromethorphan, and* ***(b)*** *plasma haloperidol levels were unchanged by in vivo* 24-hour *icv propranolol pre-treatment. v- vehicle, p- propranolol icv pre-treatments. Data are expressed as mean ± standard error of mean. Calculated using independent samples t-tests for activity and haloperidol levels.*

There was a significant inverse relationship between vacuous chewing movements and CYP2D activity in brain (Figure 16a, r=-0.49, p=0.03), and a significant positive correlation between vacuous chewing movements and brain haloperidol levels (Figure 16b, r=0.55, p=0.01).

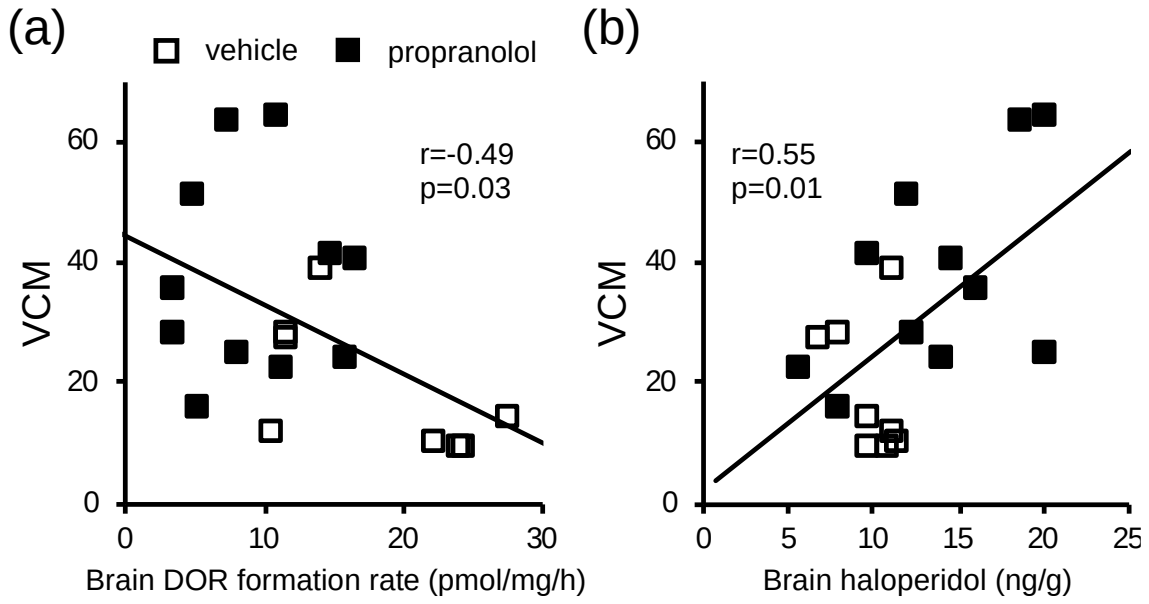


Figure 16. Chronic haloperidol-induced vacuous chewing movements (VCMs) are related to ex vivo dextrorphan (DOR) formation in the brain and brain haloperidol levels. *Vacuous chewing movements correlated negatively with (a) ex vivo CYP2D activity in the brain, as measured by dextrorphan formation from dextromethorphan, and correlated positively with (b) brain haloperidol levels.*

Vacuous chewing movements correlated with neither CYP2D activity in liver (Figure 17a, r=0.11, p=0.7) nor plasma haloperidol levels (Figure 17b, r=0.08, p=0.8).

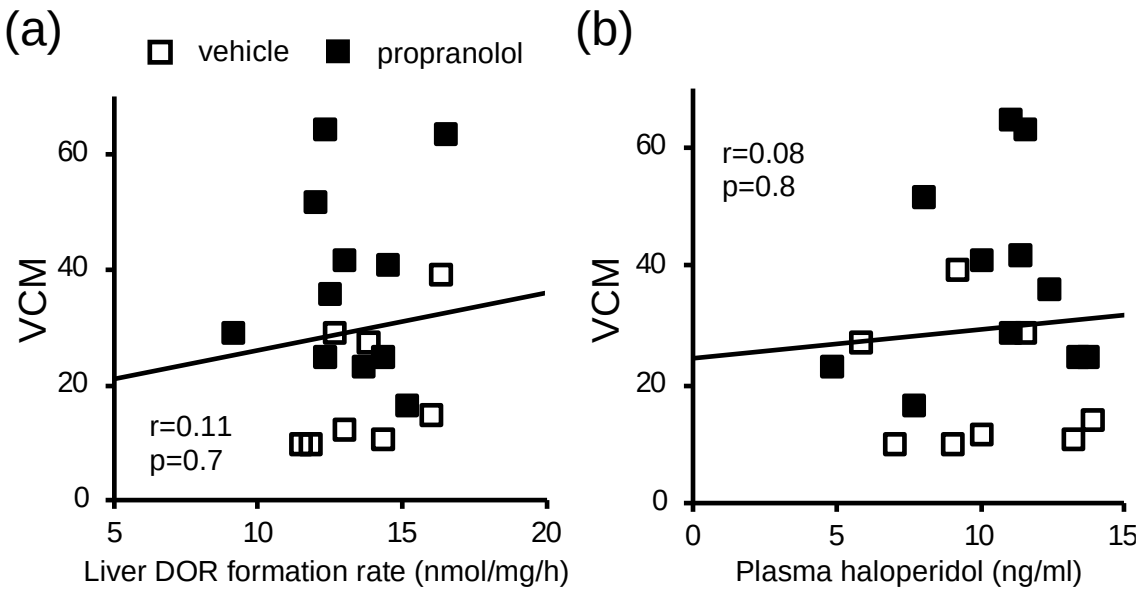


Figure 17. Chronic haloperidol-induced vacuous chewing movements (VCMs) are not related to ex vivo dextrorphan (DOR) formation in the liver and plasma haloperidol levels. *Vacuous chewing movements did not correlate with (a) ex vivo CYP2D activity in liver or (b) plasma haloperidol levels.*

3-1-E Brain lipid peroxidation was increased by inhibition of CYP2D in the brain

As tardive dyskinesia is thought to be associated with brain oxidative stress (Lister et al., 2014), we monitored the effect of inhibiting CYP2D in brain during chronic haloperidol exposure on brain lipid peroxidation. Lipid peroxidation was significantly higher in striatum and in cortex with icv propranolol pre-treatment compared to icv vehicle pre-treatment (Figure 18a), while plasma lipid peroxidation was unchanged by pre-treatment (Figure 18b). Vacuous chewing movements correlated positively with cortical lipid peroxidation (Figure 18c, r=0.54, p=0.02), and with striatal lipid peroxidation (r=0.31, p=0.1), although not significantly. Consistent with this, brain haloperidol levels correlated weakly with cortical (r=0.36, p=0.07) and striatal (r=0.43, p=0.06) lipid peroxidation. Plasma haloperidol levels correlated with plasma lipid peroxidation (r=0.44, p=0.03), but there was no relationship between vacuous chewing

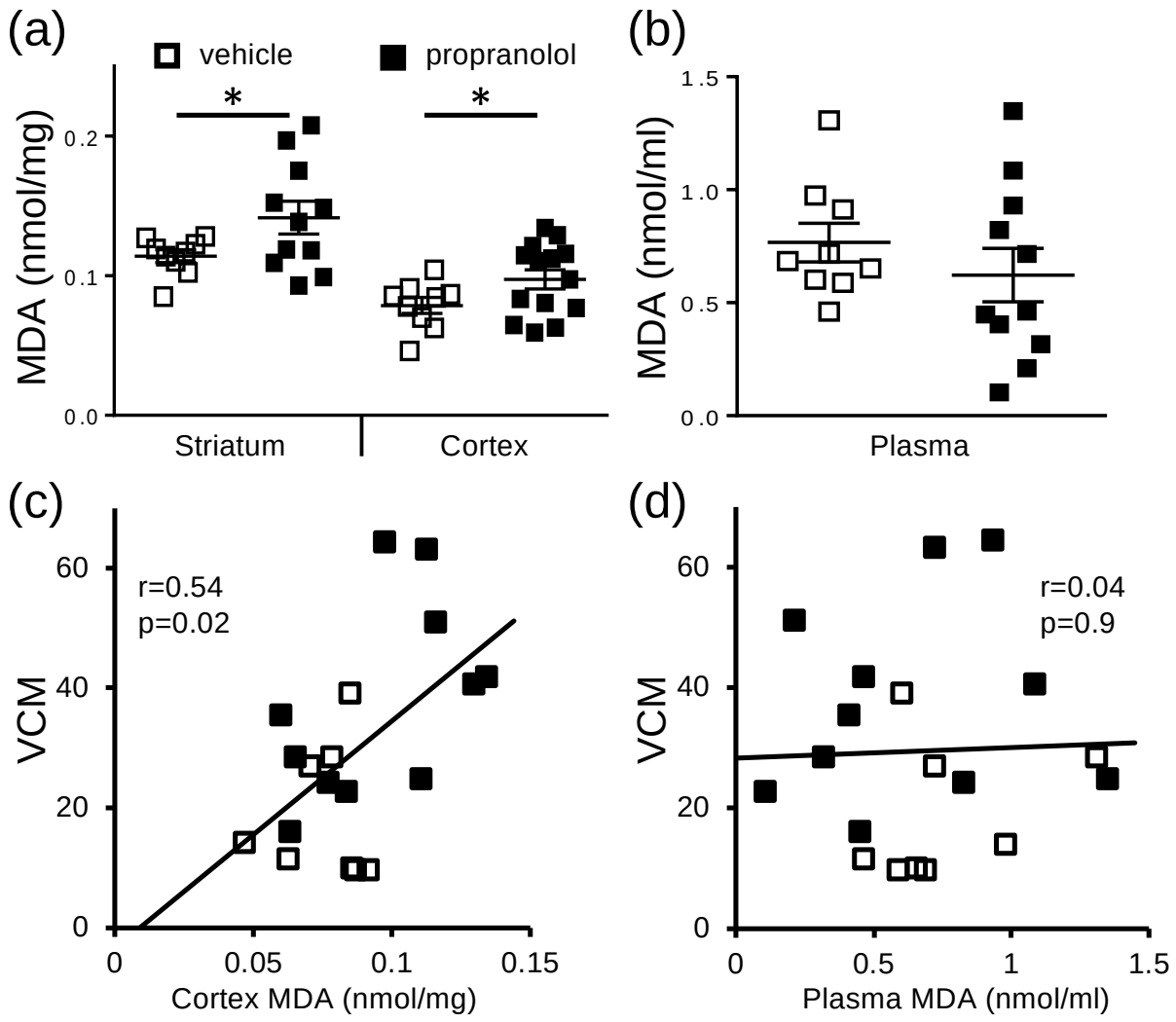


Figure 18. Chronic haloperidol-induced vacuous chewing movements (VCMs) and brain oxidative stress are related. *Lipid peroxidation, as measured by malondialdehyde (MDA) formation, was higher with in vivo* 24-hour *icv propranolol pre-treatment inhibition of* ***(a)*** *CYP2D in striatum and cortex, but not in* ***(b)*** *plasma. Vacuous chewing movements correlated with* ***(c)*** *brain, but not with* ***(d)*** *plasma lipid peroxidation. Data are expressed as mean ± standard error of mean. *p<0.05 calculated using independent samples t-test.*

movements and plasma lipid peroxidation (Figure 18d, r=0.04, p=0.9). Together, these data provide evidence that pre-treatment with CYP2D inhibitor in brain increased brain haloperidol levels, vacuous chewing movements, and brain lipid peroxidation, and suggest that brain haloperidol levels may be responsible for vacuous chewing movements.

3-1-F Summary of results

Selectively increasing and/or decreasing CYP2D activity in brain can alter the behavioural responses in rat models of acute and chronic haloperidol side-effects; these manipulations have opposite effects in the acute model relative to the chronic model. Inhibiting CYP2D in brain via *in vivo* 24-hour icv propranolol pre-treatment reduced haloperidol-induced catalepsy, a model of acute parkinsonism, while inducing CYP2D in brain via 7-day nicotine treatment increased catalepsy, suggesting a role for a neurotoxic metabolite of haloperidol formed by CYP2D in brain. In contrast, inhibiting CYP2D in brain increased chronic haloperidol-induced vacuous chewing movements, a model of tardive dyskinesia, suggesting that this is mediated by the parent compound. Inhibiting CYP2D in brain in the chronic model increased brain lipid peroxidation, which correlated with increased vacuous chewing movements, providing complementary support for an increase in neurotoxicity from higher brain haloperidol levels. Further, in both of these models, the manipulations of CYP2D activity in brain selectively altered CYP2D activity and haloperidol levels in brain but did not alter CYP2D activity in liver and haloperidol levels in plasma.

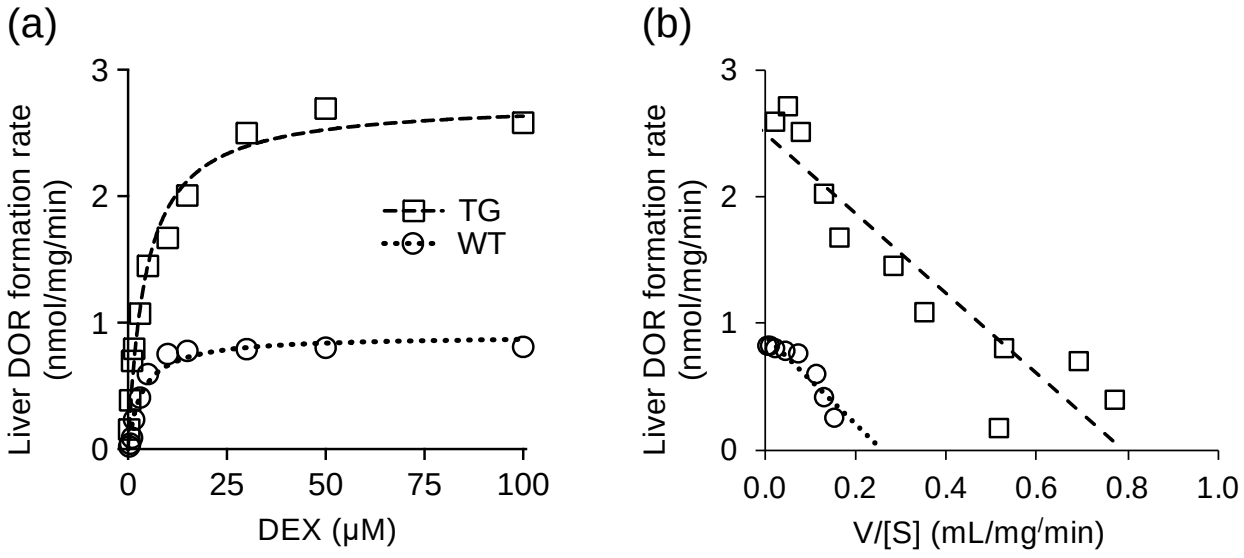
3-2 Study 2: Propranolol is a mechanism-based inhibitor of CYP2D and CYP2D6 in humanized CYP2D6-transgenic mice: effects on activity and drug response

Propranolol is a useful tool to study CYP2D as propranolol acts as an MBI of human CYP2D6 (Rowland et al., 1994; Shaw et al., 1987) and rat CYP2D (Masubuchi et al., 1991; Schneck and Pritchard, 1981). Specifically, an *in vivo* 24-hour pre-treatment with icv propranolol irreversibly inhibits CYP2D in rat brain, as demonstrated by changes in behavioural response to CYP2D substrates codeine (McMillan and Tyndale, 2015), oxycodone (McMillan et al., 2019), and haloperidol (Results of Section 3-1). To improve the understanding of the role of CYP2D6 in the human brain, we proposed to use *in vivo* 24-hour pre-treatment with icv propranolol in TG, which express both human CYP2D6 and mouse CYP2D. Before experiments such as those performed in Section 3-1 can be carried out, *in vitro* and *in vivo* ip propranolol inhibition of mouse CYP2D and human CYP2D6 expressed in mouse must first be characterized. In Section 3-2, *in vitro* propranolol was assessed as an MBI of human CYP2D6 and mouse CYP2D, inhibition and inactivation characteristics of propranolol were characterized, and the *in vivo* impact of 24-hour ip propranolol administration on drug metabolism and response was studied. Results have been published (doi: 10.1111/bph.14884).

3-2-A TG mice exhibit faster *in vitro* dextrorphan formation rate compared to WT mice

Using liver microsomes, V_{max} values of 2.8 nmol/mg/min for TG, and of 0.9 nmol/mg/min for WT, and K_m values of 4.5 µM for TG and of 3.6 µM for WT were derived from Michaelis-Menten and

Eadie-Hofstee plots (Figures 19a and 19b). The CL_{int} was estimated to be threefold faster in TG compared to WT. The kinetic parameters for each mouse line are summarized in Figure 19c.



(c)

	K_m (μM)	V_{max} (nmol/mg/min)	CL_{int} (V_{max}/K_m) (mL/mg/min)
TG	4.5 ± 0.6	2.8 ± 0.1	0.9 ± 0.2
WT	3.6 ± 0.7	0.9 ± 0.0	0.3 ± 0.1

Mean ± SD

Figure 19. TG show faster in vitro dextrorphan (DOR) formation by liver microsomes compared to WT. (a) *Michaelis–Menten and* ***(b)*** *Eadie–Hofstee plots of in vitro dextrorphan formation rate from dextromethorphan (DEX) in TG and WT liver microsomes.* ***(c)*** *Enzyme kinetic values were obtained from these plots.*

3-2-B *In vitro* propranolol irreversibly inhibits *in vitro* dextrorphan formation by mouse liver microsomes

A K_i of 35 nM for TG and of 10 nM for WT liver microsomes was observed (Figures 20a and 20b). The IC_{50} plots demonstrated that the inhibitory effect of *in vitro* propranolol on *in vitro* dextrorphan formation in TG and WT liver microsomes was increased by preincubation with NADPH. The data suggested *in vitro* propranolol inhibition of a one-site system for WT and of a

two-site system for TG liver microsomes. *In vitro* propranolol preincubation with NADPH decreased the $IC_{50,High}$ (3.8-fold) and $IC_{50,Low}$ (1.4-fold) in TG liver microsomes and decreased the IC_{50} (8.3-fold) in WT liver microsomes (Figures 20c and 20d).

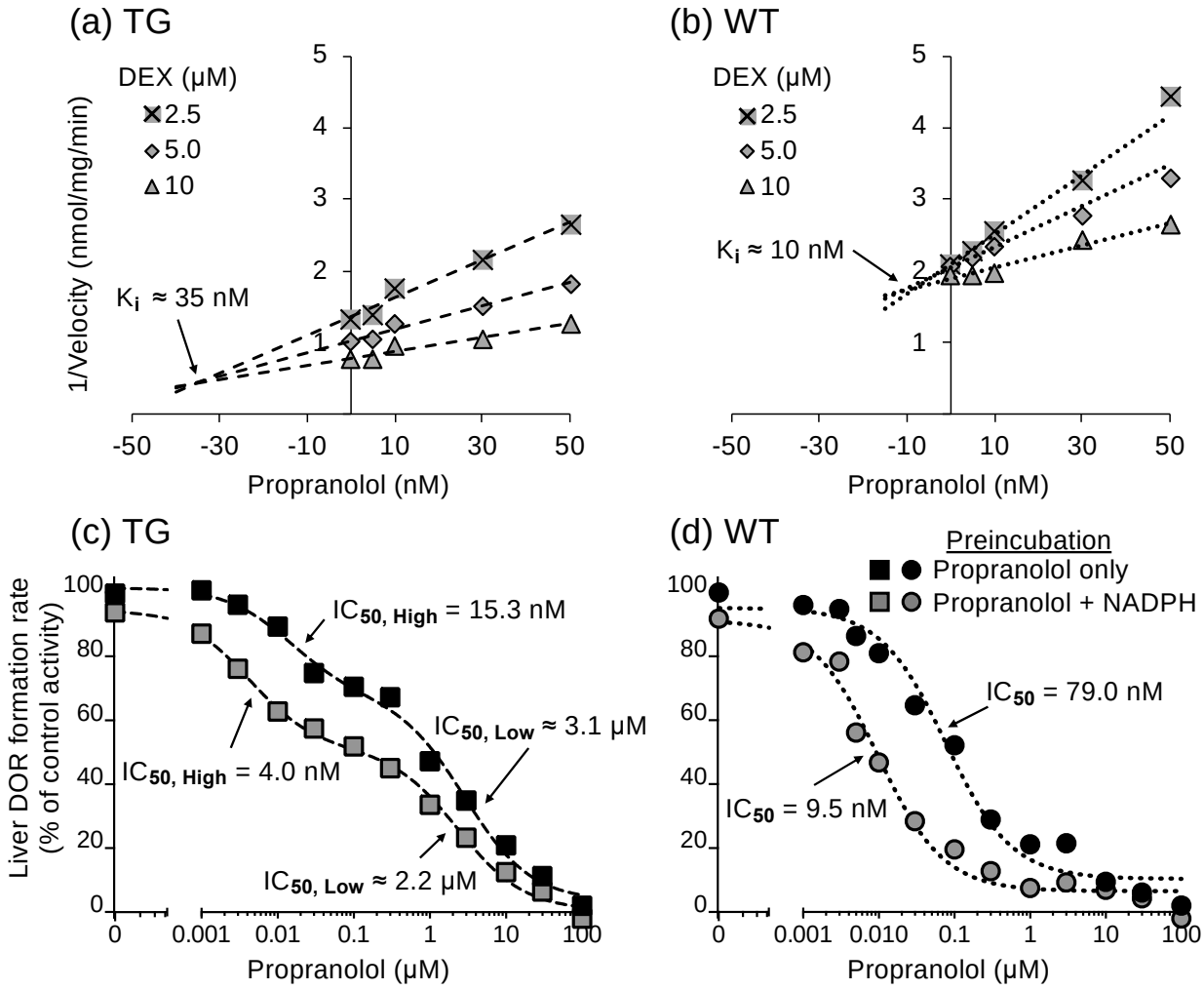


***Figure 20. In vitro propranolol inhibits in vitro dextrorphan (DOR) formation rate in TG and WT liver. (a** and **b)** Dixon and **(c** and **d)** IC50 plots of dextrorphan formation rate from dextromethorphan (DEX), by pooled **(a** and **c)** TG and **(b** and **d)** WT liver microsomes. Pooled **(a)** TG or **(b)** WT liver microsomes (0.1 mg/ml) were incubated with propranolol (0–50 nM) for 5 minutes before 2.5, 5, or 10 μM of dextromethorphan was added. Pooled **(c)** TG or **(d)** WT liver microsomes (0.1 mg/ml) were incubated with propranolol (0–100 μM), with or without NADPH, for 5 minutes before dextromethorphan (5 μM) was added. The difference in dextrorphan formation between propranolol preincubation, with and without NADPH, before the dextromethorphan was added, suggests that propranolol acts as an MBI of CYP2D in liver microsomes from **(c)** TG and **(d)** WT.*

In vitro propranolol inhibited *in vitro* dextrorphan formation rate in a concentration- and time-dependent manner for TG and WT liver microsomes (Figures 21a and 21b). This suggests that *in vitro* propranolol was an MBI of CYP2D activity in TG and WT liver. The $k_{inactivation}$ and K_I for CYP2D were 0.11 min^{-1} and 84 nM for TG, and 0.24 min^{-1} and 11 nM for WT liver microsomes, respectively (Figures 21c and 21d).

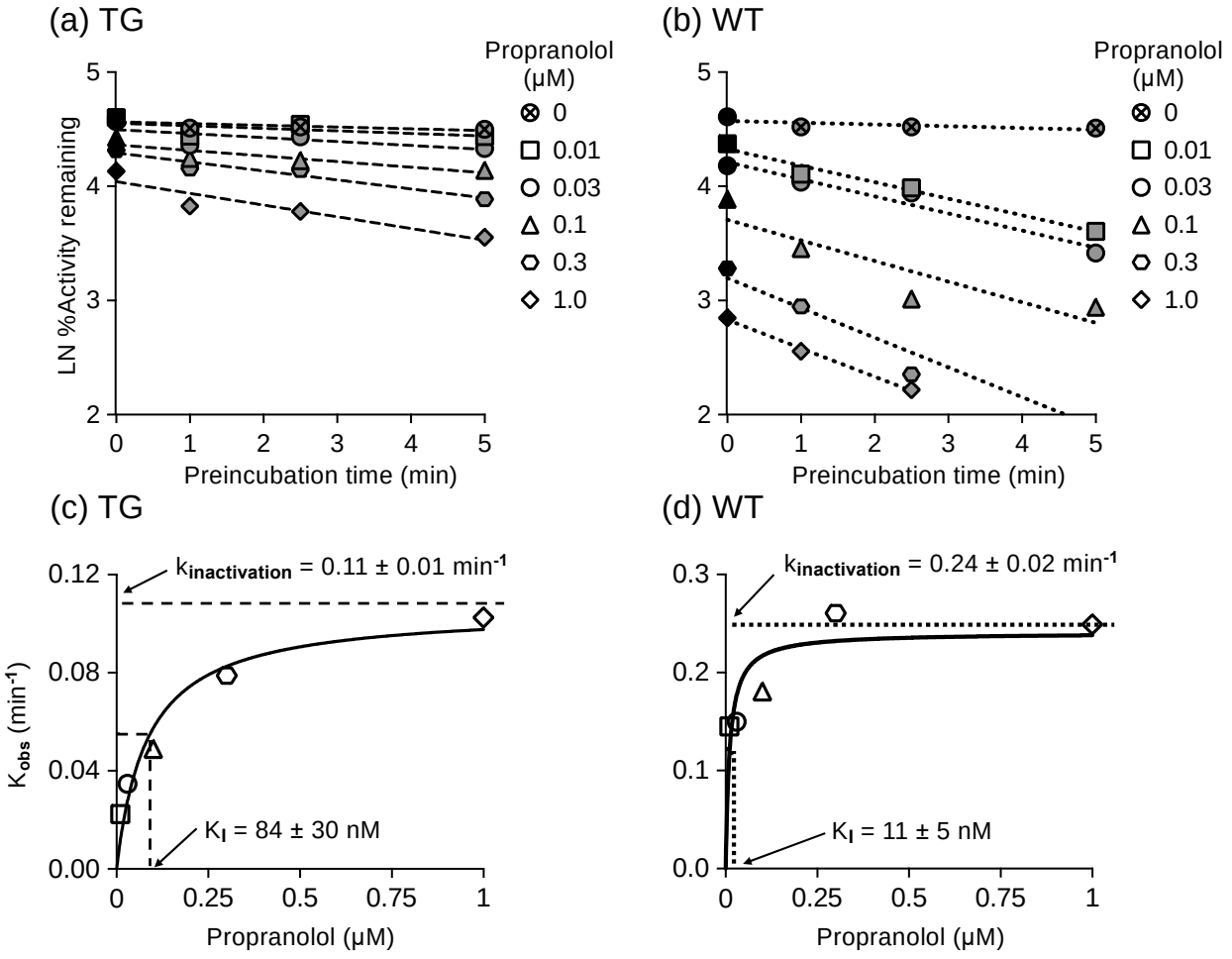


Figure 21. In vitro propranolol acts as an MBI of in vitro CYP2D activity in TG and WT liver. *In vitro propranolol (0-1 μM) inhibition of in vitro dextrorphan formation rate from dextromethorphan, a measure of CYP2D activity, by pooled* ***(a)*** *TG and* ***(b)*** *WT liver microsomes (0.1 mg/ml) was preincubation time and dose-dependent. The rate of inactivation of CYP2D (K_{obs}) by each propranolol concentration was plotted to determine $k_{inactivation}$ and K_I of* ***(c)*** *TG and* ***(d)*** *WT.*

In vitro propranolol preincubation with NADPH decreased the IC_{50} in human liver microsomes (1.8-fold) compared to preincubation with propranolol alone (Figure 22a). *In vitro* propranolol also inhibited *in vitro* dextrorphan formation rate in a concentration- and time-dependent manner for

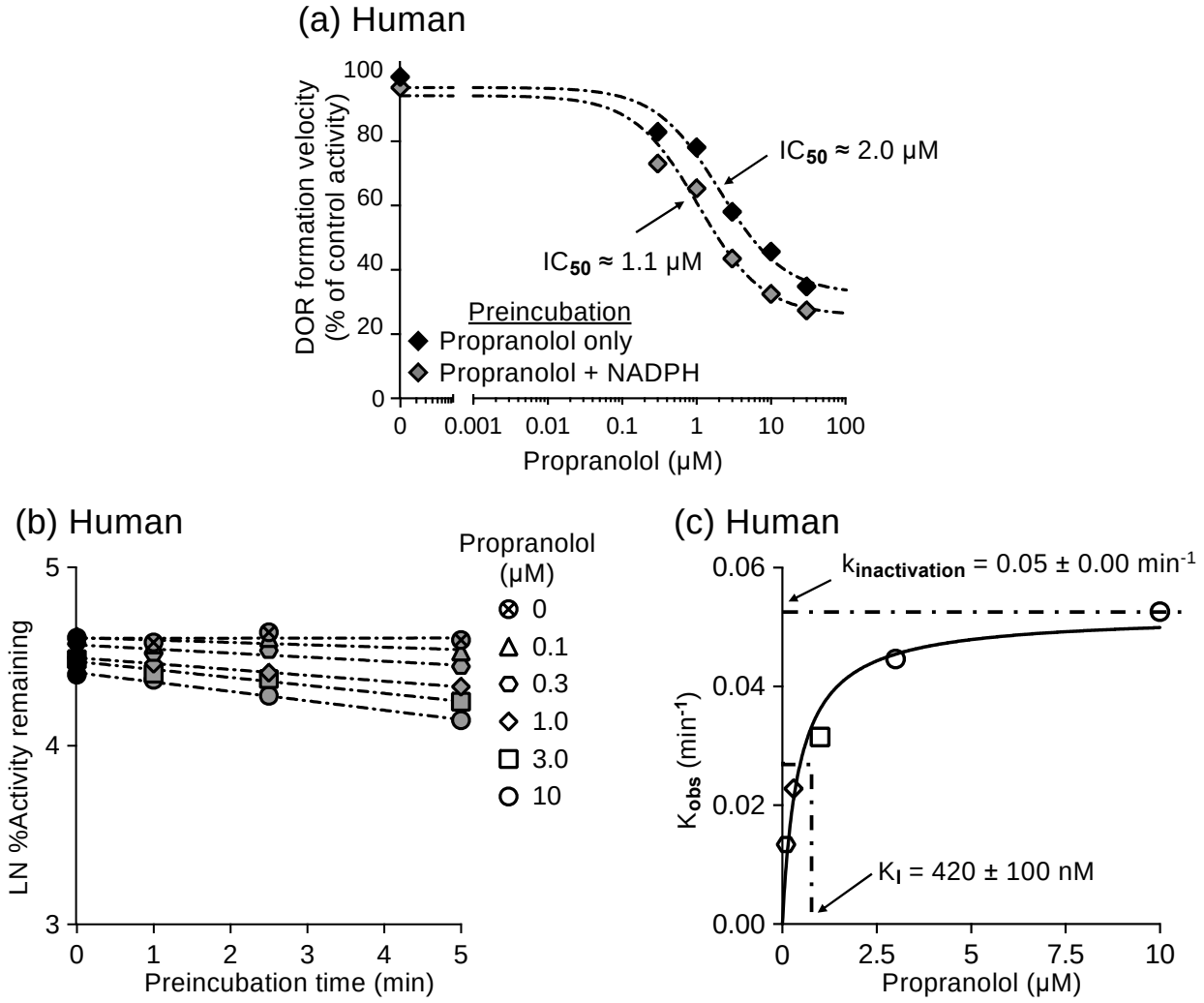


Figure 22. In vitro propranolol acts as an MBI of in vitro CYP2D6 activity in human liver. ***(a)*** *Pooled human liver microsomes (0.1 mg/ml) were preincubated with propranolol (0–30 µM) for 5 minutes before dextromethorphan (5 µM) was added. The difference in in vitro dextrorphan formation rate, a measure of CYP2D6 activity, between propranolol preincubation with and without NADPH, before dextromethorphan was added, suggests that propranolol acts as an MBI of CYP2D6 in liver microsomes from humans.* ***(b)*** *Propranolol (0–10 µM) inhibition of in vitro dextrorphan formation rate by pooled human liver microsomes (0.1 mg/ml) was preincubation time and dose-dependent.* ***(c)*** *The rate of inactivation of CYP2D6 (K_{obs}) by each propranolol concentration was plotted to determine $k_{inactivation}$ and K_I.*

human liver microsomes (Figure 22b), and the $k_{inactivation}$ and K_I for CYP2D6 were 0.05 min^{-1} and 420 nM, respectively (Figure 22c).

3-2-C *In vivo* 24-hour pre-treatment with ip propranolol reduces *in vivo* plasma dextrorphan/dextromethorphan ratio and *ex vivo* liver dextrorphan formation in TG and WT

After the *in vivo* 24-hour pre-treatment with ip propranolol (versus vehicle), the plasma dextrorphan/dextromethorphan was significantly reduced in TG (by 26%, Figure 23a) and WT (by 33%, Figure 23b). Using the liver microsomes from these *in vivo* 24-hour ip propranolol pre-treated mice (versus saline), *ex vivo* dextrorphan formation rate was significantly reduced in TG (by 22%, Figure 23c) and WT mice (by 25%, Figure 23d). The long-lasting (greater than 24-hour) inhibition by *in vivo* propranolol of subsequent *in vivo* and *ex vivo* dextrorphan formation suggests that *in vivo* propranolol pre-treatment irreversibly inhibits CYP2D in TG and WT mice, consistent with the inhibition previously observed from *in vitro* propranolol treatment (Figures 21a and 21b).

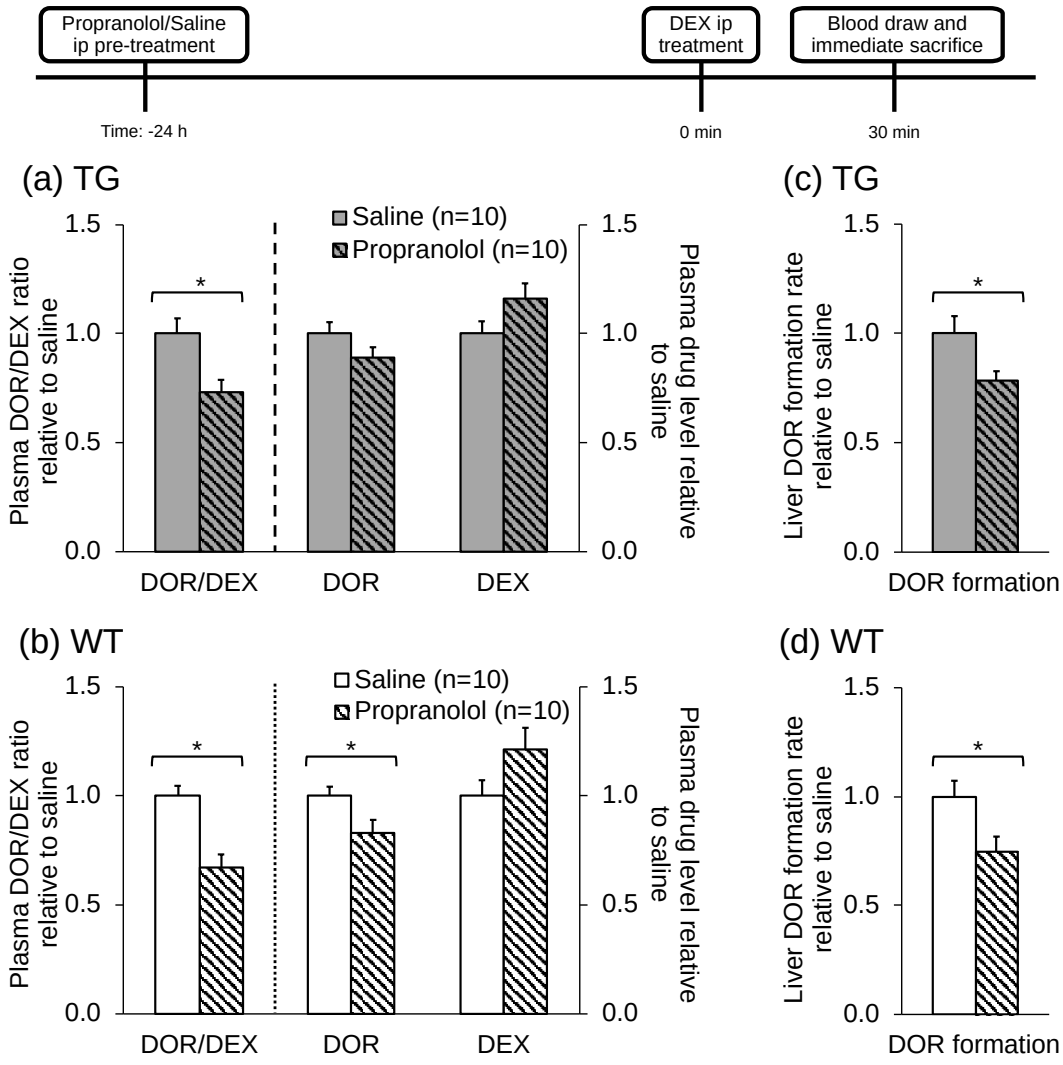


Figure 23. In vivo 24-hour pre-treatment with ip propranolol decreased in vivo plasma dextrorphan/dextromethorphan (DOR/DEX) ratio and decreased ex vivo liver dextrorphan formation in TG and WT. *After in vivo 24-hour pre-treatment with either ip propranolol (20 mg/kg) or saline, mice were given an ip injection of 30 mg/kg of dextromethorphan and blood was collected via saphenous vein 30 minutes after dextromethorphan injection. The in vivo plasma dextrorphan/dextromethorphan ratio, plasma dextrorphan and plasma dextromethorphan levels in* ***(a)*** *TG and* ***(b)*** *WT mice were assessed. The ex vivo dextrorphan formation by liver microsomes (0.1 mg/ml) prepared from pre-treated* ***(c)*** *TG and* ***(d)*** *WT was assessed after incubation with dextromethorphan (5 µM) for 10 minutes. Data presented are expressed as mean plus standard error of mean of individual animals normalized to the saline pre-treated group, within the same mouse line. *p<0.05, **p<0.01, ***p<0.001 calculated using two-tailed, unpaired t test on the raw data.*

3-2-D *In vivo* 24-hour pre-treatment with ip propranolol increases *in vivo* plasma haloperidol levels and haloperidol-induced catalepsy

Plasma haloperidol levels were significantly increased by *in vivo* 24-hour pre-treatment with ip propranolol compared to saline pre-treatment within animals, in both TG (by 19% and 28%, at 60 and 180 min, respectively, Figure 24a; $AUC_{60\text{-}180}$ by 22%, Figure 24c) and WT mice (by 30% and 37%, at 60 and 180 min, respectively, Figure 24b; $AUC_{60\text{-}180}$ by 32%, Figure 24d). Similarly, the mean catalepsy scores at 120 minutes post-haloperidol injection were significantly increased by 24-hour ip pre-treatment with propranolol, compared to saline pre-treatment within animals, in both TG (by 31%, Figure 24e) and WT (by 35%, Figure 24f).

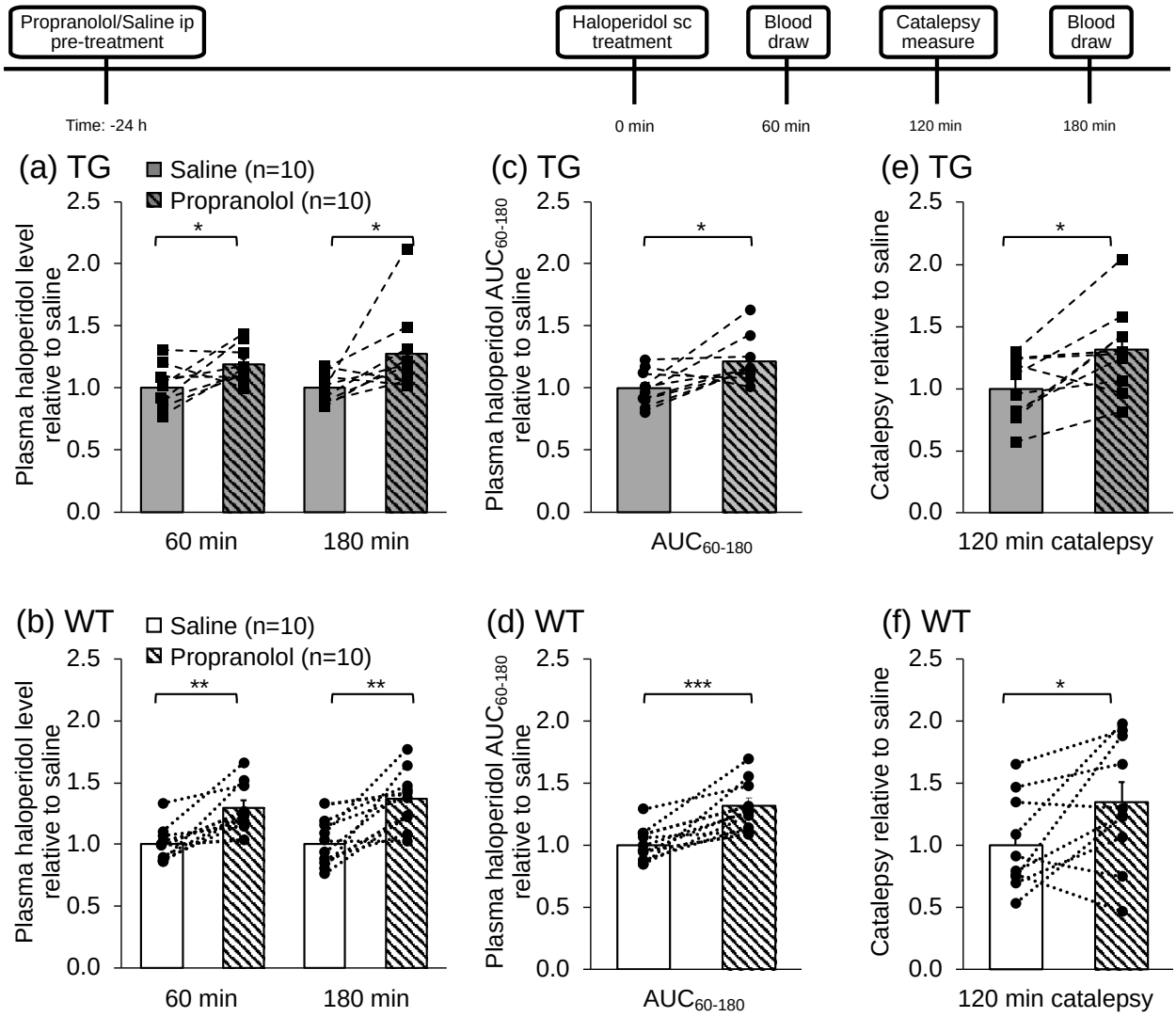


Figure 24. In vivo plasma haloperidol, plasma haloperidol $AUC_{60–180}$, and acute haloperidol-induced catalepsy are increased (within animals) in TG and WT after in vivo 24-hour pre-treatment with ip propranolol (versus saline). *After in vivo 24-hour pre-treatment with either ip propranolol or saline,* ***(a, c,*** *and* ***e)*** *TG and (b, d, and* ***f)*** *WT were given a sc injection of 0.1 mg/kg of haloperidol,* ***(a*** *and* ***b)*** *plasma haloperidol levels were assessed at 60 and 180 minutes after the haloperidol injection, and* ***(c*** *and* ***d)*** *plasma haloperidol AUC from 60 to 180 minutes was derived.* ***(e*** *and* ***f)*** *Catalepsy was tested at 120 minutes after haloperidol injection. Data are expressed as mean plus standard error of mean of each individual animal normalized to the saline pre-treated group, within the same mouse line. Pre-treatments were given 2 weeks apart. *p<0.05, **p<0.01, ***p<0.001 calculated using two-tailed, paired t test on the raw data.*

3-2-E Summary of results

We have shown that TG (versus WT) have faster *in vitro* dextrorphan formation by liver microsomes, and that *in vitro* propranolol irreversibly inhibits *in vitro* dextrorphan formation by liver microsomes of TG, WT, and humans. Mice given *in vivo* 24-hour pre-treatment with ip propranolol had significantly reduced *in vivo* plasma dextrorphan/dextromethorphan ratio in both TG and WT, and significantly reduced *ex vivo* dextrorphan formation by liver microsomes prepared from these pre-treated animals. After showing that *in vivo* propranolol given ip acts as an MBI of *in vivo* and *ex vivo* CYP2D-mediated dextromethorphan metabolism to dextrorphan, haloperidol was used to confirm the effects of propranolol mechanism-based inhibition of CYP2D by measuring *in vivo* plasma haloperidol levels and response to haloperidol. Mice given *in vivo* 24-hour pre-treatment with ip propranolol had significantly higher *in vivo* plasma haloperidol levels and haloperidol-induced catalepsy. These data indicate that *in vitro* and *in vivo* propranolol exerts mechanism-based inhibition of hepatic CYP2D in TG and WT, and that this inhibition is sufficient to alter drug-induced behaviour (catalepsy).

3-3 Study 3: Human CYP2D6 is functional in brain *in vivo*: evidence from humanized CYP2D6 transgenic mice

The results from Section 3-2 suggest that *in vitro* and *in vivo* propranolol is an MBI of human CYP2D6 (in humans and TG), and of mouse CYP2D (in TG and WT). To improve our understanding of the impact of human CYP2D in brain on drug response, we used a similar inhibition approach as described in Section 3-1. We expected that *in vivo* propranolol administered directly into the mouse brain (icv) would also act as an MBI of both CYP2D6 (in TG brain) and mouse CYP2D (in TG and WT brain). Initial experiments revealed that *in vivo* icv propranolol acted as an MBI of CYP2D6 in TG brain, but not of mouse CYP2D in WT brain. Although unexpected, this provided a novel strategy to study human CYP2D6 in brain alone (leaving mouse CYP2Ds in brain and liver, and hepatic CYP2D6 unaffected) in a live animal. Results have been published (doi: 10.1007/s12035-020-01896-4).

3-3-A TG exhibit higher i*n vivo* brain dextrorphan/dextromethorphan ratio and faster *ex vivo* brain dextrorphan formation rate compared to WT

The *in vivo* brain dextrorphan/dextromethorphan ratio was significantly higher (243%, Figure 25a) and *ex vivo* dextrorphan formation rate by brain membranes was significantly faster (137%, Figure 25b) in TG compared to WT. This is consistent with TG expressing human CYP2D6, in addition to mouse CYP2D, in TG brain, compared with WT that express mouse CYP2D alone.

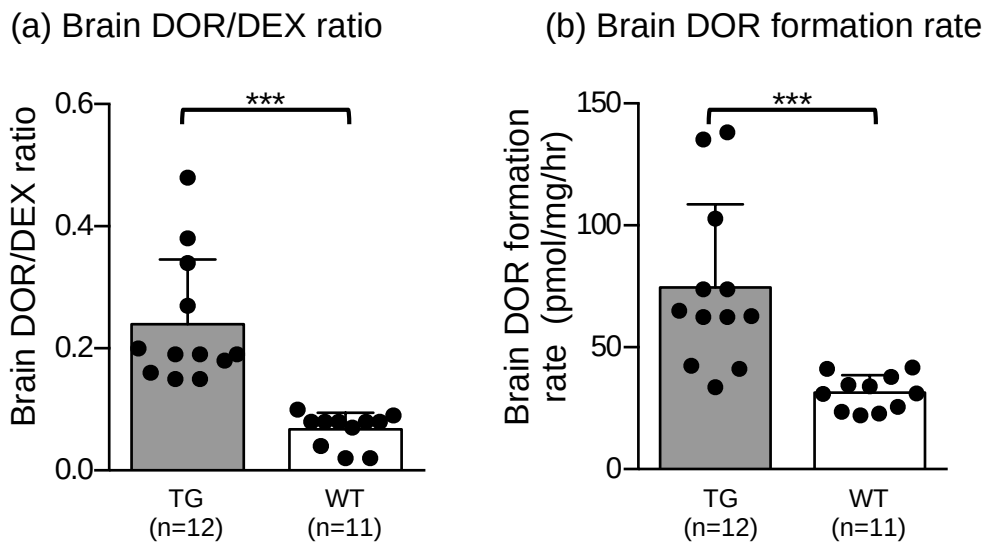


Figure 25. TG had higher in vivo brain dextrorphan/dextromethorphan (DOR/DEX) ratio and faster ex vivo brain dextrorphan formation rate in brain membranes compared to WT. (a) *Brain dextrorphan/dextromethorphan ratio and* ***(b)*** *brain dextrorphan formation rate are shown for TG and WT. *p<0.05, **p<0.01, ***p<0.001 calculated using a two-tailed, unpaired t-test.*

3-3-B *In vivo* 4-hour pre-treatment with icv propranolol decreased the *in vivo* brain dextrorphan/dextromethorphan ratio in TG and WT, and decreased the *ex vivo* brain dextrorphan formation rate in TG, but not in WT

After the *in vivo* 4-hour pre-treatment with icv propranolol (versus vehicle) (Figures 26a and 27a), the *in vivo* brain dextrorphan/dextromethorphan ratio was significantly decreased in both TG (22% decrease) and in WT (47% decrease) (Figure 26b). This suggests inhibition of CYP2D *in vivo* in brains of both mouse lines. After the *in vivo* 4-hour pre-treatment with icv propranolol (versus vehicle), the *ex vivo* brain dextrorphan formation rate was significantly decreased (39%) in brain membranes from TG, but the icv propranolol pre-treatment had no effect on *ex vivo* brain dextrorphan formation rate in WT (Figure 26c). This suggests that *in vivo* propranolol (given as a 4-hour icv pre-treatment) acted as an MBI of the human CYP2D6 in TG brain but acted as a competitive inhibitor of mouse CYP2D in TG and WT brain.

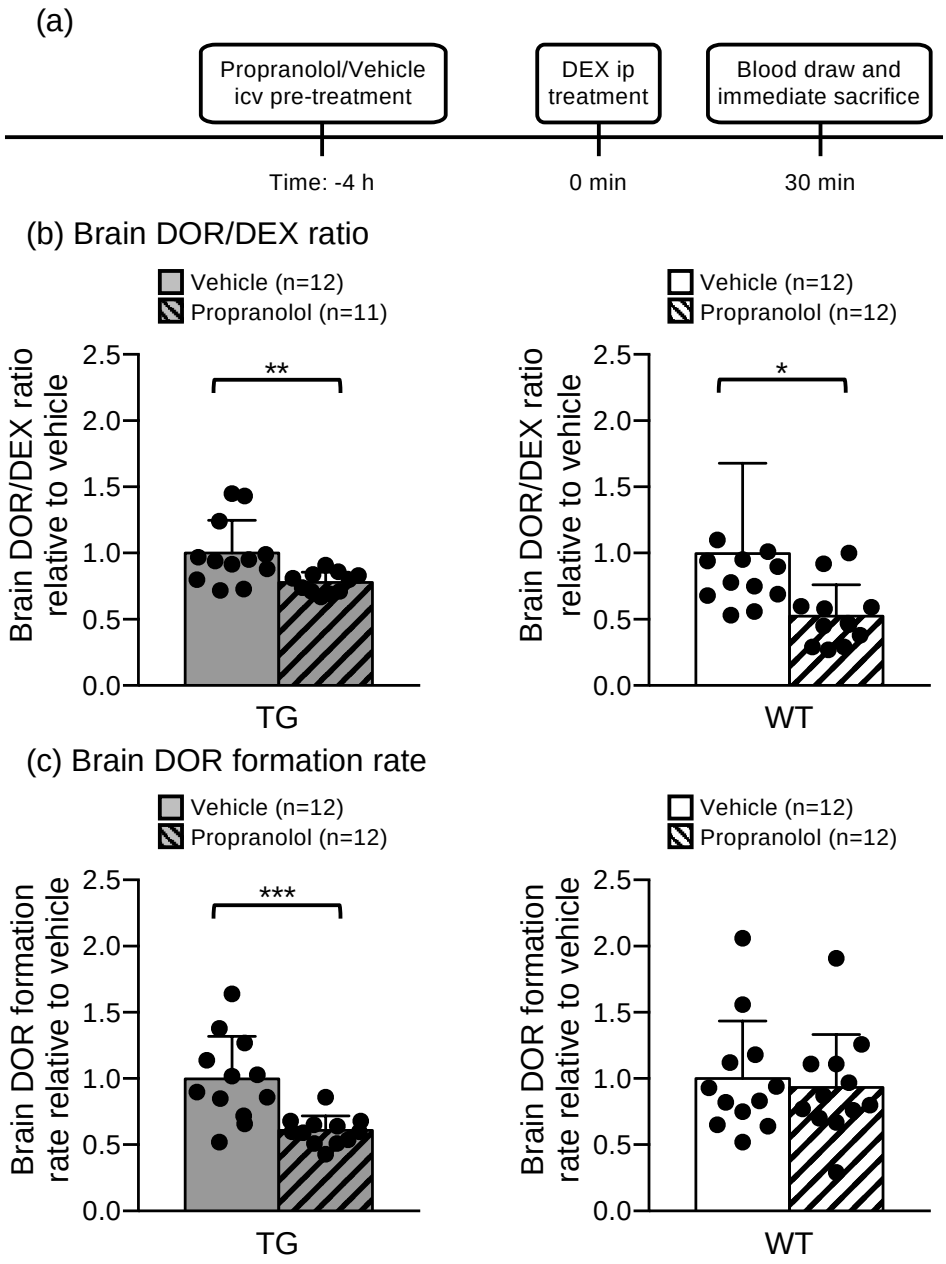


Figure 26. The in vivo 4-hour pre-treatment with icv propranolol decreased the in vivo brain dextrorphan/dextromethorphan (DOR/DEX) ratio in TG and WT, and decreased the ex vivo dextrorphan formation rate in brain membranes in TG, but not in WT. (a) *Experimental design.* ***(b)*** *Brain dextrorphan/dextromethorphan ratio and* ***(c)*** *brain dextrorphan formation rate are shown for TG (left side) and WT (right side). The data is illustrated as the mean, relative to the vehicle pre-treatment group within mouse line, plus standard deviation. The brain dextrorphan/dextromethorphan data from one TG animal was excluded due to dextromethorphan levels being 9-fold above the mean brain dextromethorphan level. Data analysis was run on all remaining animals. However, the brain dextrorphan/dextromethorphan data from one WT animal was excluded from this figure (value 3.09). *p<0.05, **p<0.01, ***p<0.001 calculated using a two-tailed, unpaired t-test.*

As expected, after the *in vivo* 4-hour pre-treatment with icv propranolol (versus vehicle), there was no difference in the *in vivo* plasma dextrorphan/dextromethorphan ratio (Figure 27b) nor was there

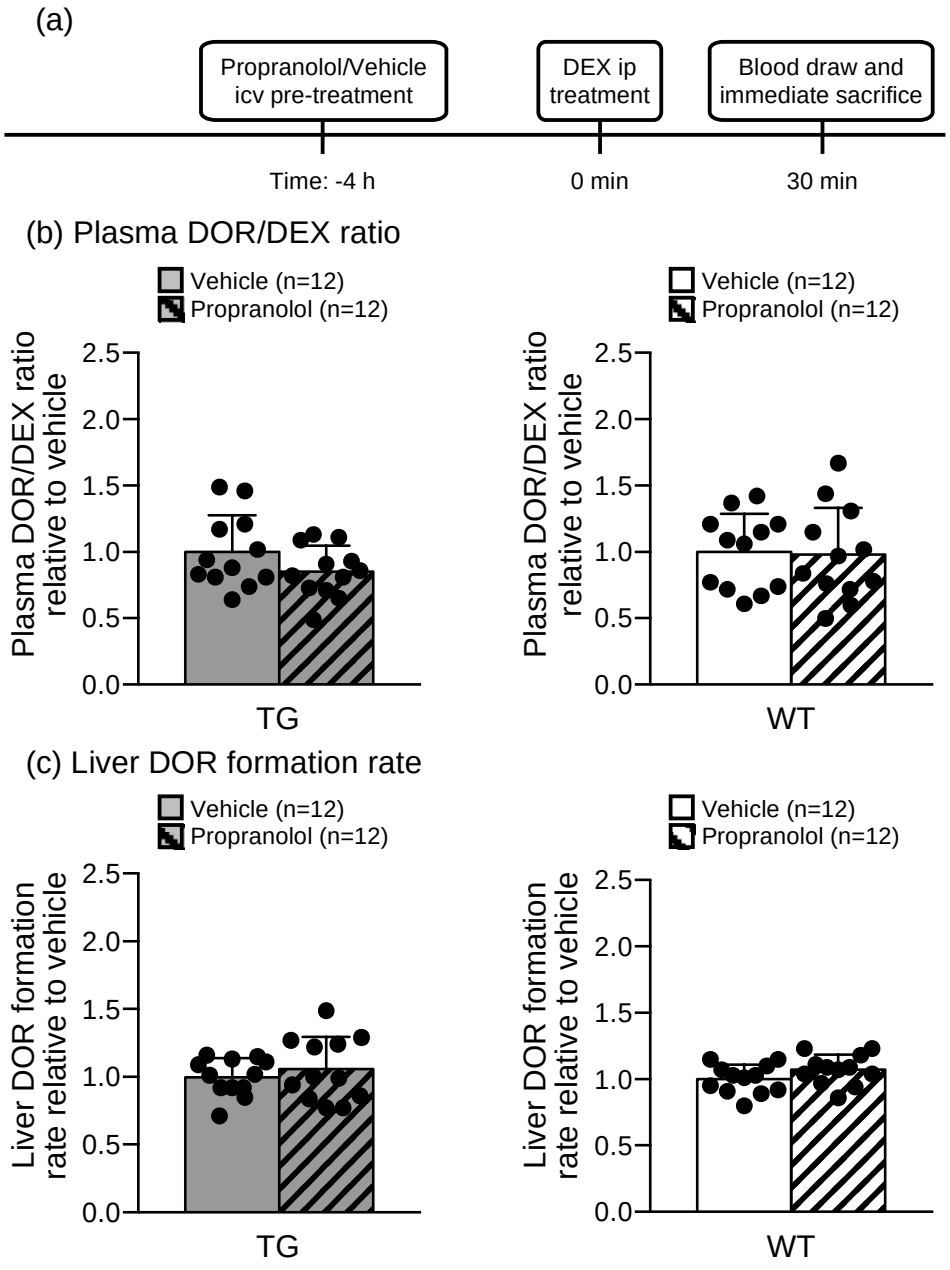


Figure 27. The in vivo 4-hour pre-treatment with icv propranolol did not change the in vivo plasma dextrorphan/dextromethorphan (DOR/DEX) ratio nor did it change the ex vivo dextrorphan formation rate in liver microsomes in TG and WT. (a) *Experimental design.* ***(b)*** *Plasma dextrorphan/dextromethorphan ratio, and* ***(c)*** *liver dextrorphan formation rate are shown for TG (left side) and WT (right side). The data is illustrated as the mean, relative to the vehicle pre-treatment group within mouse line, plus standard deviation. Calculated using a two-tailed, unpaired t-test.*

a difference in the *ex vivo* liver dextrorphan formation rate (Figure 27c) in either mouse line. Thus, the *in vivo* 4-hour pre-treatment with icv propranolol did not result in propranolol entering the systemic system and inhibiting liver CYP2D *in vivo* (plasma dextrorphan/dextromethorphan ratio) or *ex vivo* (liver dextrorphan formation rate) in either TG or WT.

3-3-C *In vivo* 24-hour pre-treatment with icv propranolol decreased the *in vivo* brain dextrorphan/dextromethorphan ratio and the *ex vivo* brain dextrorphan formation rate in TG, but not in WT

After the *in vivo* 24-hour pre-treatment with icv propranolol (versus vehicle) (Figures 28a and 29a), the *in vivo* brain dextrorphan/dextromethorphan ratio was significantly decreased (34%) in TG, but not in WT (Figure 28b). The *ex vivo* brain dextrorphan formation rate was also significantly decreased (37%) in TG, but not in WT (Figure 28c). These data suggest that, following this longer pre-treatment interval, *in vivo* propranolol (given as a 24-hour icv pre-treatment) acted as an MBI of human CYP2D6 in TG brain but not of mouse CYP2D in TG and WT brain.

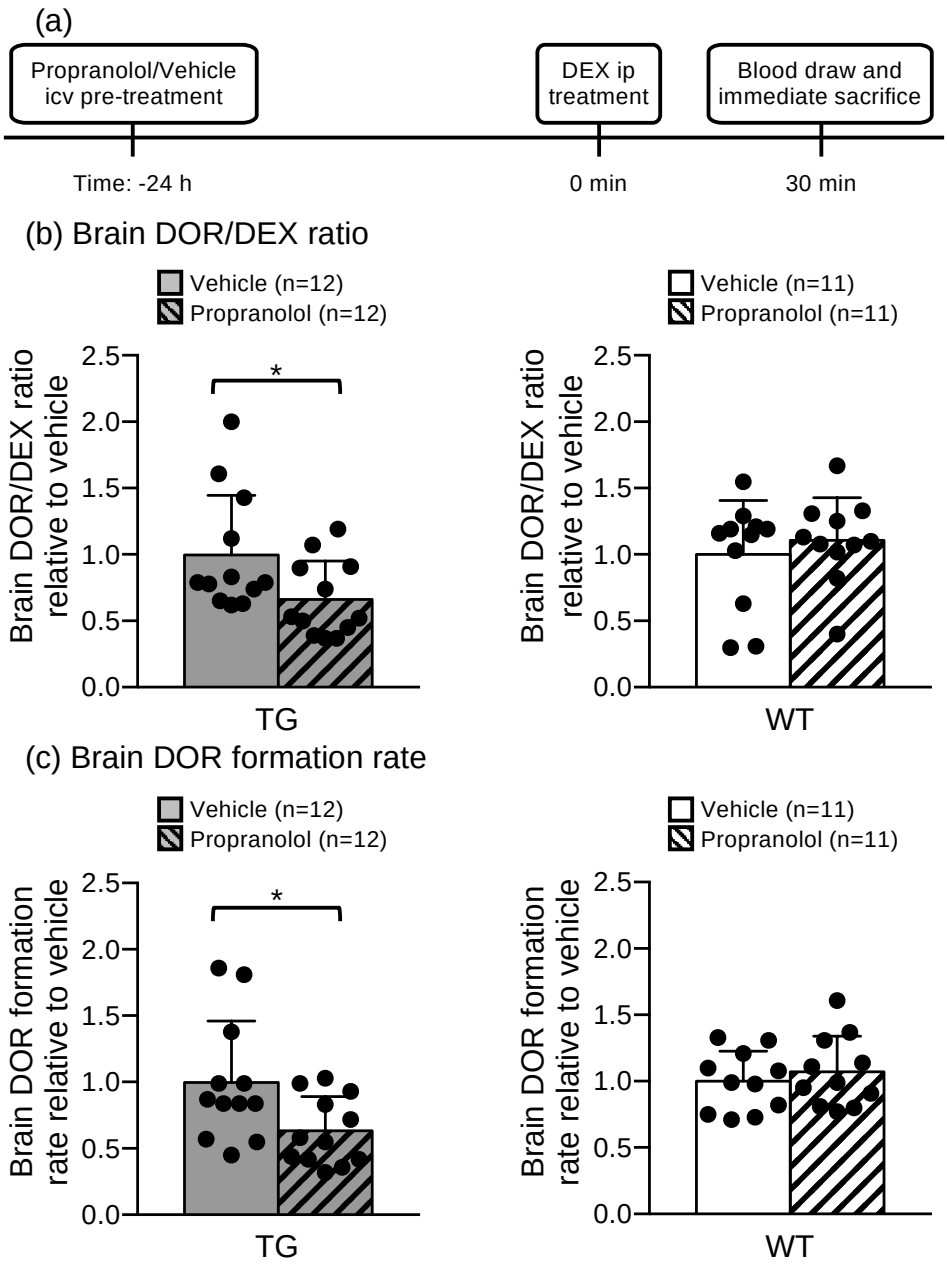


Figure 28. The in vivo 24-hour pre-treatment with icv propranolol decreased the in vivo brain dextrorphan/dextromethorphan (DOR/DEX) ratio and the ex vivo dextrorphan formation rate in brain membranes in TG, but not in WT. (a) *Experimental design.* ***(b)*** *Brain dextrorphan/dextromethorphan ratio and* ***(c)*** *brain dextrorphan formation rate are shown for TG (left side) and WT (right side). The data is illustrated as the mean relative to the vehicle pre-treatment group within mouse line, plus standard deviation. *p<0.05 calculated using a two-tailed, unpaired t-test.*

Again, as expected, after the *in vivo* 24-hour pre-treatment with icv propranolol (versus vehicle), there was no difference in the *in vivo* plasma dextrorphan/dextromethorphan ratio (Figure 29b) nor

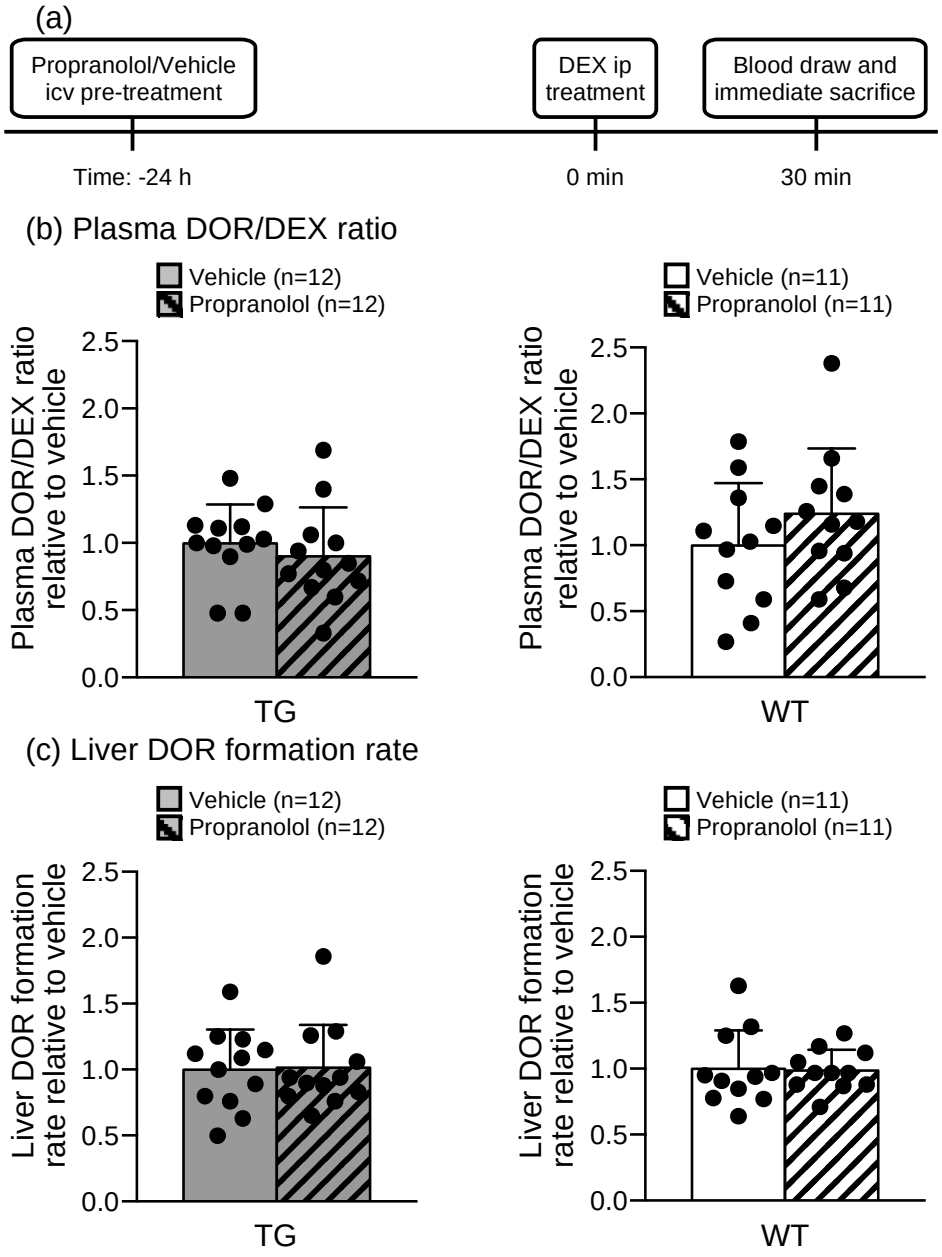


Figure 29. The in vivo 24-hour pre-treatment with icv propranolol did not change the in vivo plasma dextrorphan/dextromethorphan (DOR/DEX) ratio nor did it change the ex vivo dextrorphan formation rate in liver microsomes in TG and WT. (a) *Experimental design.* ***(b)*** *Plasma dextrorphan/dextromethorphan ratio and* ***(c)*** *liver dextrorphan formation rate are shown for TG (left side) and WT (right side). The data is illustrated as the mean relative to the vehicle pre-treatment group within mouse line, plus standard deviation. Calculated using a two-tailed, unpaired t-test.*

was there a difference in the *ex vivo* liver dextrorphan formation rate (Figure 29c) in either mouse line. Thus, the *in vivo* 24-hour pre-treatment with icv propranolol did not result in propranolol entering the systemic system and inhibiting CYP2D in the liver *in vivo* (plasma dextrorphan/dextromethorphan ratio) or *ex vivo* (liver dextrorphan formation rate) for TG and WT.

3-3-D Haloperidol-induced catalepsy response was higher in TG compared to WT

Catalepsy was tested following different haloperidol doses and time points post-haloperidol injection to select parameters that allowed for the detection of changes in catalepsy. Catalepsy reached a stable level by 60 minutes, which was maintained to 180 minutes post-haloperidol injection (Figure 30a) as observed previously in rats (see Figure 4a) and mice (Ionov and Severtsev, 2012; Nishchal et al., 2014). There was a main effect of dose and time for TG (F(2,10)

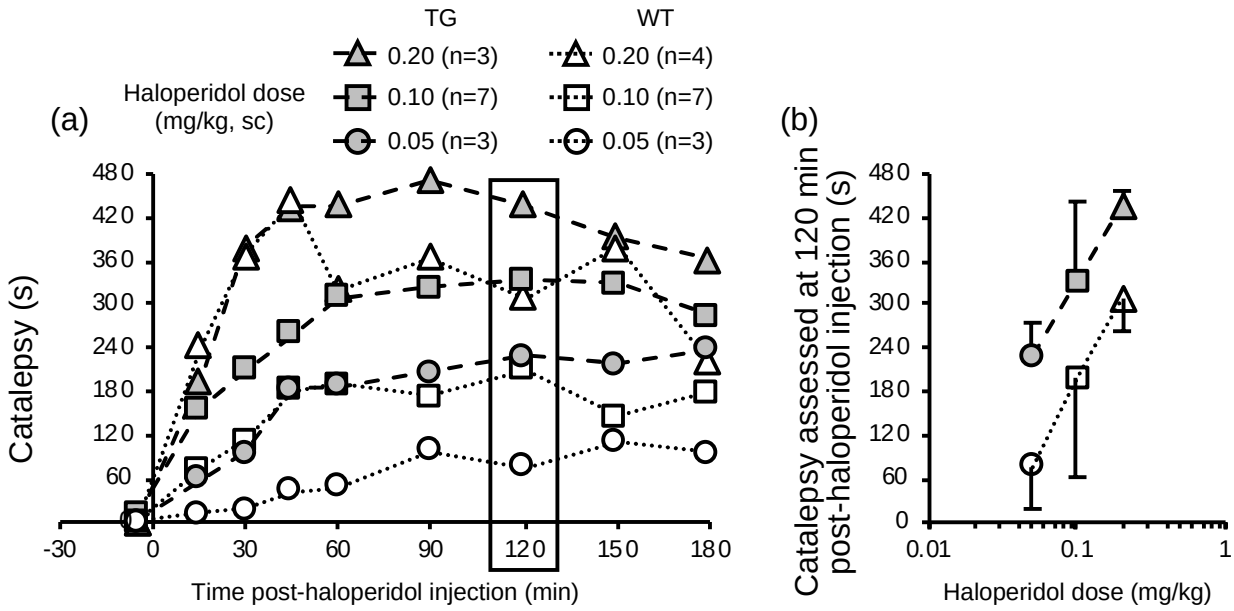


Figure 30. TG experienced longer catalepsy compared to WT. (a) *Haloperidol-induced catalepsy is assessed.* ***(b)*** *Catalepsy at 120 minutes post-haloperidol (boxed in* ***a)*** *is in the linear portion of the dose response curve for each line. The* ***(b)*** *data is illustrated as the mean catalepsy plus (for TG) or minus (for WT) standard deviation. Effect of dose (two-way ANOVA, $F_{2,21}$ = 10.50, $p < .001$) and mouse line (two-way ANOVA, $F_{1,21}$ = 15.35, $p < .001$).*

= 27.11, $p < 0.001$; $F(8, 80) = 48.78$, $p < 0.001$) and for WT ($F(2,11) = 25.94$, $p < 0.001$; $F(8.88) = 11.66$, $p < 0.001$). Catalepsy from 0.1 mg/kg haloperidol was significantly higher than catalepsy from 0.05 mg/kg and significantly lower than catalepsy from 0.2 mg/kg haloperidol in TG ($p < 0.01$ and $p < 0.01$, respectively) and in WT ($p < 0.05$ and $p < 0.001$, respectively). At 120 minutes post-haloperidol catalepsy, there was a main effect of dose ($F(2,21) = 10.50$, $p < 0.01$) and mouse line ($F(1,21) = 15.35$, $p < 001$). Catalepsy at 120 minutes after 0.1 mg/kg haloperidol was significantly higher than after 0.05 mg/kg haloperidol and significantly lower than after 0.2 mg/kg haloperidol for TG ($p < 0.05$ and $p < 0.05$, respectively); the following experiments tested catalepsy at 120 minutes after a 0.1 mg/kg haloperidol injection. We also collected blood at times before and after the catalepsy to ensure no impact on plasma haloperidol levels after the *in vivo* icv 24-hour propranolol pre-treatment. TG had significantly higher (67%) catalepsy compared to WT (Figure 30b).

3-3-E *In vivo* 24-hour pre-treatment with icv propranolol decreased catalepsy in TG, but not in WT

The *in vivo* 24-hour pre-treatment with icv propranolol (versus vehicle) (Figures 31a, 32a, and 33a) did not change mean baseline catalepsy response in TG (7s versus 9s, respectively) or in WT (3s versus 3s, respectively). After the *in vivo* 24-hour pre-treatment with icv propranolol (versus vehicle), catalepsy was significantly decreased (33%) in TG, but not in WT (Figure 31b). This is consistent with *in vivo* 24-hour pre-treatment with icv propranolol inhibiting CYP2D-mediated haloperidol metabolism in TG, but not in WT, brain.

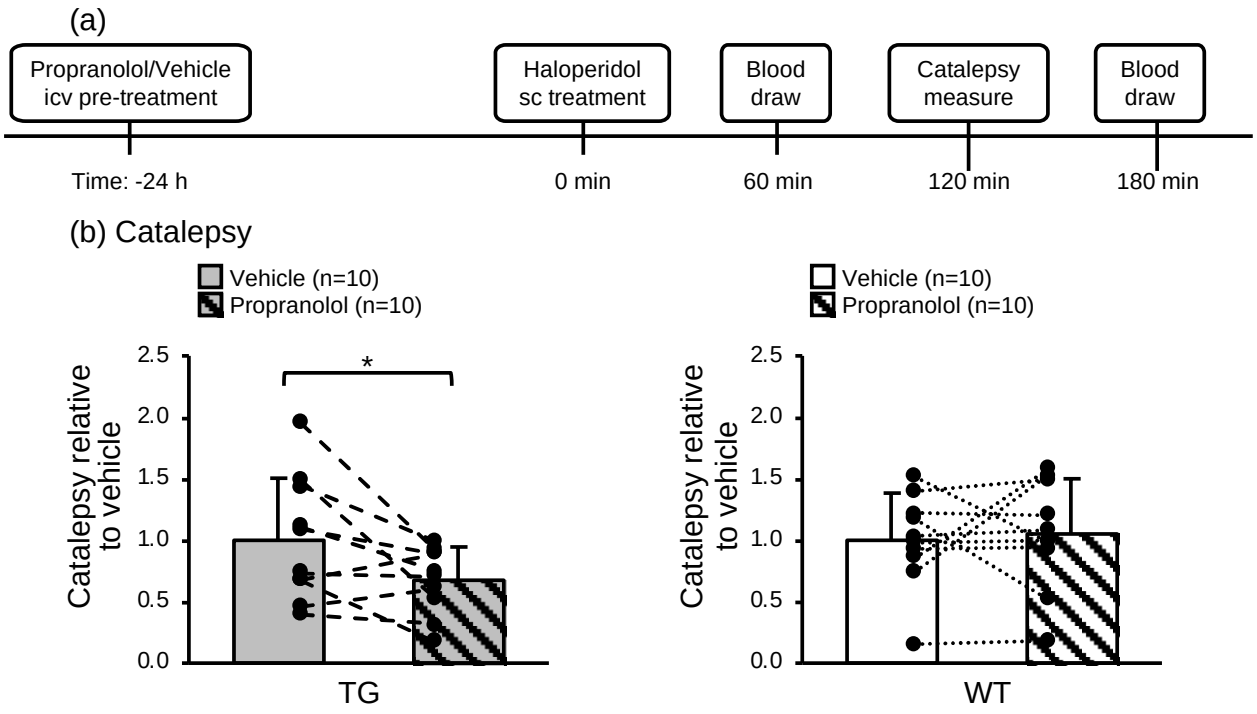


Figure 31. The in vivo 24-hour pre-treatment with icv propranolol decreased the haloperidol-induced catalepsy in TG, but not in WT. (a) *Experimental design.* ***(b)*** *Mean catalepsy is shown for TG and WT. Lines represent data from individual mice crossed by pre-treatment. The data is illustrated as the mean relative to the vehicle pre-treatment group within mouse line, plus standard deviation. *p<0.05 calculated using a two-tailed, paired t-test.*

As expected, after the *in vivo* 24-hour pre-treatment with icv propranolol (versus vehicle), there was no difference in plasma haloperidol $AUC_{60\text{-}180}$ (Figure 32b) or plasma haloperidol levels at 60 and at 180 minutes post-haloperidol injection (Figures 33a and 33b) in either TG or WT. This indicates that *in vivo* 24-hour icv propranolol pre-treatment did not result in propranolol entering the systemic system and inhibiting liver CYP2D-mediated haloperidol metabolism in either TG or WT.

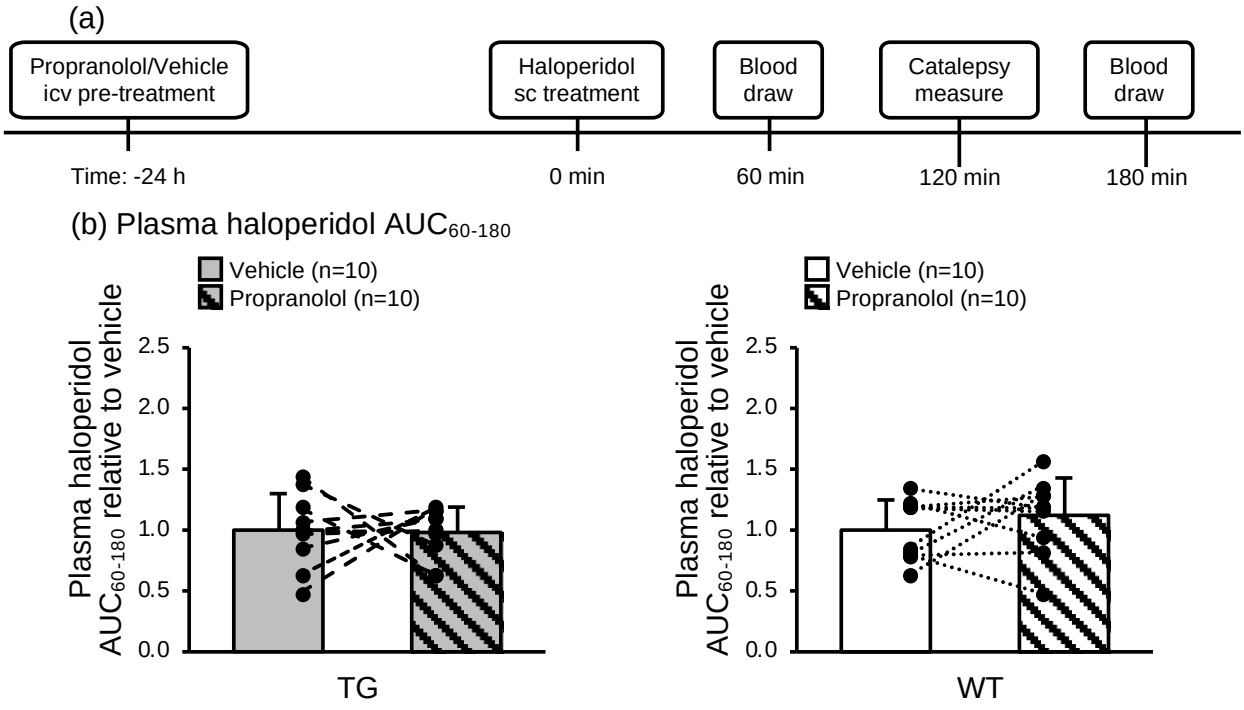


Figure 32. The in vivo 24-hour pre-treatment with icv propranolol had no impact on in vivo plasma haloperidol AUC from 60 to 180 minutes post-haloperidol injection. (a) *Experimental design.* ***(b)*** *Plasma haloperidol $AUC_{60\text{-}180}$ is shown for TG and WT. Lines represent data from individual mice crossed by pre-treatment. The data is illustrated as the mean relative to the vehicle pre-treatment group within mouse line, plus standard deviation. Calculated using a two-tailed, paired t-test.*

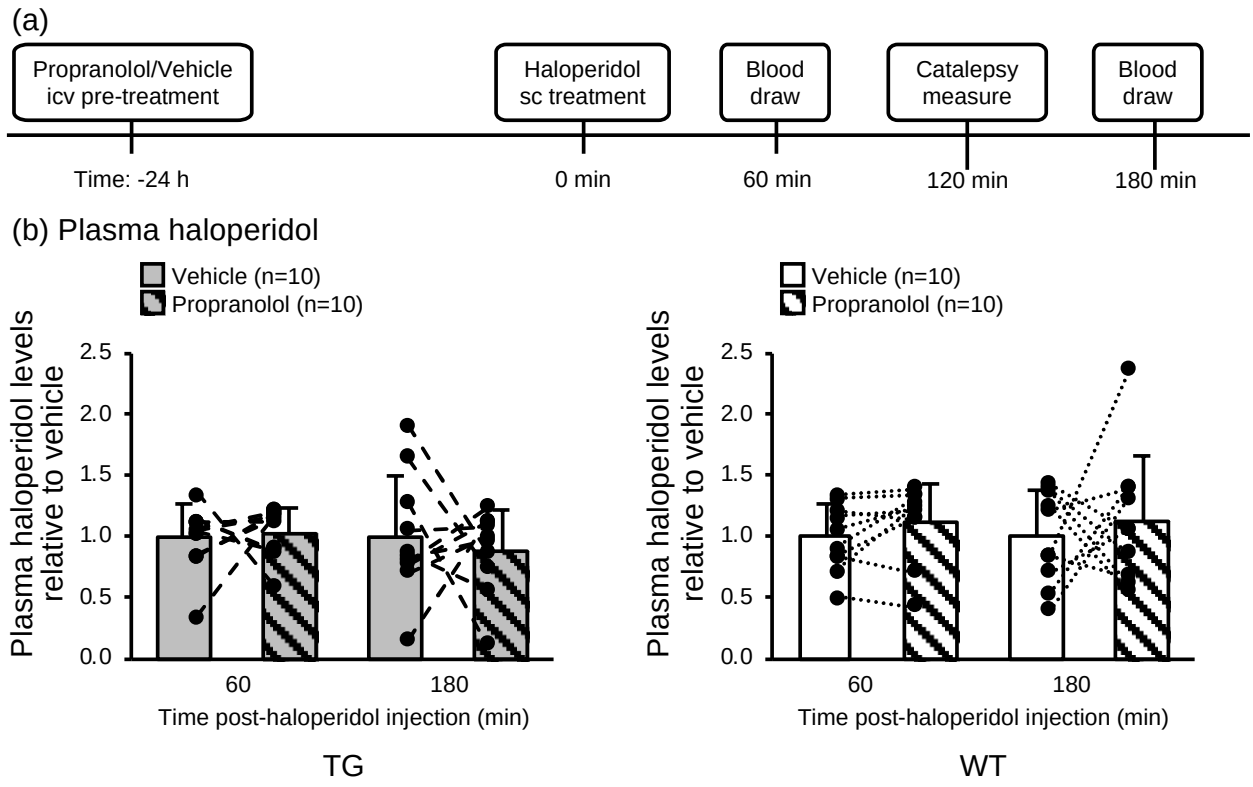


Figure 33. The in vivo 24-hour pre-treatment with icv propranolol had no impact on in vivo plasma haloperidol levels at 60- and 180-minutes post-haloperidol injection. (a) *Experimental design.* ***(b)*** *Mean plasma haloperidol levels at 60- and 180-minutes post-haloperidol injection are shown for TG and WT. Lines represent data from individual mice crossed by pre-treatment. The data is illustrated as the mean relative to the vehicle pre-treatment group within mouse line, plus standard deviation. Calculated using a two-tailed, paired t-test.*

3-3-F Summary of results

The *in vivo* 4-hour pre-treatment with icv propranolol decreased the *in vivo* brain dextrorphan/dextromethorphan ratio and decreased the *ex vivo* brain dextrorphan formation rate in TG. Conversely, in WT, the *in vivo* 4-hour pre-treatment with icv propranolol decreased the *in vivo* brain dextrorphan/dextromethorphan ratio but did not decrease the *ex vivo* brain dextrorphan formation rate. The *in vivo* 24-hour pre-treatment with icv propranolol decreased the *in vivo* brain dextrorphan/dextromethorphan ratio and decreased the *ex vivo* brain dextrorphan formation rate in TG, and had no effect on either in WT. Inhibition of *ex vivo* brain dextrorphan formation in TG

and not in WT after both *in vivo* icv pre-treatment intervals suggests that propranolol acts as an MBI of human CYP2D6 in TG brain, but not of mouse CYP2D in TG and WT brain. The *in vivo* brain dextrorphan ratio, *ex vivo* brain dextrorphan formation rate, and catalepsy at 120 minutes were higher in TG compared to WT, consistent with the additional contribution of CYP2D6 in TG versus WT. The *in vivo* 24-hour pre-treatment with icv propranolol (versus vehicle) decreased catalepsy response in TG, but not in WT, demonstrating that CYP2D6 in brain is functional *in vivo* with sufficient enzymatic activity to alter drug response. In all experiments, *in vivo* plasma drug levels and *ex vivo* liver enzymatic activity were unaltered by *in vivo* icv inhibitor pre-treatments.

4 Discussion

4-1 Study 1: Rat brain CYP2D enzymatic metabolism alters acute and chronic haloperidol side-effects by different mechanisms

Herein we examined how selectively inducing or inhibiting CYP2D in rat brain alters acute and chronic haloperidol side effects. First, we determined that acute haloperidol-induced catalepsy was decreased after inhibiting CYP2D in brain, and increased after inducing CYP2D in brain, suggesting that catalepsy may be related to a neurotoxic haloperidol metabolite formed in the brain by CYP2D. Second, we determined that chronic haloperidol-induced vacuous chewing movements were increased after inhibiting CYP2D in brain, suggesting that vacuous chewing movements may be related to the parent compound haloperidol. An alternative interpretation is that vacuous chewing movements may be related to a neurotoxic haloperidol metabolite catalyzed by a different enzyme (i.e. not CYP2D). Following inhibition of CYP2D in the brain, parent haloperidol levels would remain high and an alternative metabolism could form a neurotoxic metabolite. Thus, we have not only demonstrated a significant role in the brain for variable CYP2D-mediated metabolism in both acute and chronic haloperidol side-effects, but we have also provided new, contrasting mechanistic insights into these side-effects: a CYP2D-formed haloperidol metabolite in the brain contributes to acute side-effects and haloperidol itself (or a non-CYP2D metabolite) contributes to chronic side-effects. See Section 3-1 for detailed results.

4-1-A Haloperidol acute vs chronic side effects

The apparently opposing roles for CYP2D in brain in the acute vs chronic haloperidol treatment likely reflect mechanistic differences in the underlying neurochemistry in acute versus chronic

haloperidol-induced side-effects. Acute parkinsonism, modelled by catalepsy, is associated with rapid and somewhat prolonged changes; catalepsy is initiated within minutes, and extends beyond 90 minutes (Figure 4). This rapid acute response suggests, for example, changes in cell signaling. In contrast, chronic tardive dyskinesia, modelled by vacuous chewing movements, develops more slowly, plateauing after weeks of exposure (Figure 12), and thus seems more likely to be associated with longer-term neuroadaptive changes. Another contrast is that acute side-effects respond to anticholinergic drugs, while few treatment options are available for tardive dyskinesia (Lockwood and Remington, 2015). Together we conclude that elevated CYP2D in brain appears to increase risk for acute, and protect against chronic, antipsychotic side-effects.

4-1-B Impact of manipulating CYP2D in the brain

In this study, catalepsy was decreased by *in vivo* 24-hour pre-treatment with icv propranolol (Figures 6a and 7) and increased by 7-day nicotine pre-treatment (Figures 6b and 7). This increase following nicotine was reversed by subsequent *in vivo* 24-hour pre-treatment with icv propranolol (Figure 7), providing evidence that these changes in catalepsy are likely mediated through inhibition and induction of CYP2D in brain, respectively. This suggests that a CYP2D-mediated metabolite in the brain may be responsible for catalepsy in this model of acute antipsychotic side-effects. Haloperidol can be metabolized to the neurotoxic pyridinium ions 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-pyridinium (HPP^+) and 4-(4-chlorophenyl)-1-[4-(fluorophenyl)-4-hydroxybutyl]-pyridinium ($RHPP^+$) (Subramanyam et al., 1990); both can be formed by CYP2D (Shin et al., 2001), as well as CYP3A (Fang et al., 2001). Both HPP^+ and $RHPP^+$ are structurally related and have similar neurotoxic activities to 1-methyl-4-phenylpyridinium (MPP^+), which causes a parkinsonian syndrome in humans and animals

(Wright et al., 1998). Other haloperidol metabolites (for example from dealkylation and reductase pathways) are not known to be neurotoxic (Avent et al., 1996). These haloperidol pyridinium ion metabolites are found in tissues of patients, baboons, and rodents treated with haloperidol (Avent et al., 1996; Eyles et al., 1997; Subramanyam et al., 1990), and may contribute to catalepsy in the acute rodent model. When given peripherally to mice, HPP^+ does not cross the blood-brain barrier, suggesting that it is formed by central haloperidol metabolism (Usuki et al., 2002); consistent with this, mouse brain tissues can metabolize haloperidol to HPP^+ *ex vivo* (Usuki et al., 2002). CYP3A can also activate haloperidol to HPP^+, $RHPP^+$, and other potentially neurotoxic metabolites, and may contribute to haloperidol-induced neurotoxicity. However propranolol does not inhibit CYP3A (Turpeinen et al., 2006), and nicotine does not induce rat CYP3A in brain or liver (Stamou et al., 2015). Thus, *in vivo* 24-hour icv propranolol pre-treatment may reduce catalepsy by inhibiting the CYP2D-mediated formation of HPP^+ in brain, or another unidentified, neurotoxic metabolite(s), while nicotine pre-treatment may increase catalepsy by inducing the CY2D-mediated formation of this metabolite(s) in brain.

Nicotine can induce CYP2D in brain, but not liver, of rodents and monkeys (Mann et al., 2008; Yue et al., 2008), and smokers have higher CYP2D6 in brain (Mann et al., 2008), but not liver (Funck-Brentano et al., 2005), than non-smokers. While few reports separate the effects of smoking on acute and chronic antipsychotic-induced side-effects, there has been at least one report that acute antipsychotic-induced parkinsonism was more prevalent in smokers (Wagner et al., 1988). This is consistent with the increase in catalepsy after nicotine pre-treatment observed here (Figures 6b and 7).

4-1-C Implications for antipsychotic side-effects

Typical antipsychotics, such as haloperidol, are used extensively in the acute setting, for example in emergency rooms for immediate management of aggressive behaviour and psychosis (Powney et al., 2012; Siddiqi et al., 2016), and in the ICU for managing delirium (Barbateskovic et al., 2016). Many antipsychotics, both typical and atypical, are metabolized by CYP2D (Pouget et al., 2014). Having high CYP2D in brain, through genetics (i.e. *CYP2D6* UMs) or smoking, may potentially increase the risk for acute side-effects from a wide range of CYP2D-metabolized antipsychotics, not only from haloperidol. A postulated therapeutic approach to reduce acute side-effects may be the co-use of a CYP2D inhibitor during initial antipsychotic dosing.

In contrast, in the model of chronic side-effects, vacuous chewing movements (Figure 12), brain oxidative stress (Figure 18a), and brain (Figure 16b), but not plasma (Figure 17b), haloperidol levels were increased with inhibition of CYP2D in brain. This suggests that tardive dyskinesia is associated with higher brain haloperidol parent drug levels. This also suggests either an effect of the parent antipsychotic, haloperidol, or alternative rerouting of haloperidol to neurotoxins such as those created by CYP3A. Long term use of antipsychotic drugs is associated with neuroadaptive changes in humans and rodents, including increased brain oxidative stress (Andreazza et al., 2015; Bishnoi et al., 2008; Burger et al., 2005; Cho and Lee, 2013; Lister et al., 2017; Lister et al., 2014). Oxidative stress may occur for a number of reasons including increased dopamine turnover or increased glutamatergic transmission (Cho and Lee, 2013). Consistent with previous findings in pre-clinical models of tardive dyskinesia, we observed that higher brain haloperidol levels, after inhibition of CYP2D in brain, were associated with increased vacuous chewing movements (Figure 16b) and increased brain lipid peroxidation (Figure 18c). In addition, similar to the findings of Lister et al. (2017), we observed that brain

haloperidol and oxidative stress levels were associated with vacuous chewing movements among individual animals (Figure 18c). More broadly, this suggests that increased metabolic inactivation of antipsychotics, seen here by CYP2D, may reduce brain parent drug levels and potentially reduce risk for tardive dyskinesia associated with chronic treatment.

Smoking is generally associated with reduced risk of tardive dyskinesia (Winterer, 2010), and it has been shown previously that nicotine treatment significantly reduced haloperidol-induced vacuous chewing movements (Bordia et al., 2012). One possible neuroprotective role of nicotine is through increasing CYP2D6 metabolic activity in brain, resulting in reduced brain haloperidol levels. Human *CYP2D6* EMs have higher CYP2D6 in brain and liver and have lower plasma haloperidol levels than PMs (van der Weide and van der Weide, 2015); despite often receiving higher doses of haloperidol, they are at lower risk for tardive dyskinesia than PMs (MacNeil and Muller, 2016). Together with our current data, this supports the premise that having higher CYP2D6 in brain, either from being a *CYP2D6* EM or from smoking, may be protective against tardive dyskinesia. These findings, that tardive dyskinesia is likely associated with higher brain haloperidol parent drug levels, if extended to all antipsychotics, suggests that using the minimal effective dose is likely the best approach.

Metabolism by CYPs in brain is a novel mechanism that may contribute to the variability among individuals in their drug response. We have shown that variable CYP2D activity in brain alters haloperidol side-effects in rats, adding to the expanding list of drug-induced responses shown to be affected by metabolism within the brain (Garcia et al., 2015; McMillan et al., 2019; McMillan and Tyndale, 2015). Of note, CYP2D in brain influenced haloperidol response in opposing directions in models of acute and chronic side-effects. The data suggest a role for a CYP2D-derived antipsychotic metabolite in brain contributing to acute side-effects, and for brain levels

of the antipsychotic itself contributing to chronic side-effects. Differential rates of CYP2D-mediated metabolism of antipsychotics in brain may explain some of the variability in patients' risk for developing side-effects; an improved understanding of this source of inter-individual variability may improve prediction, and reduction, of these risks.

4-2 Study 2: Propranolol is a mechanism-based inhibitor of CYP2D and CYP2D6 in humanized CYP2D6-transgenic mice: effects on activity and drug response

Herein, we showed that TG (versus WT) have faster *in vitro* hepatic dextrorphan formation rate. We determined that *in vitro* propranolol decreases *in vitro* CYP2D6-mediated dextrorphan formation rate in TG, WT, and human liver microsomes. We also determined that *in vivo* 24-hour ip propranolol pre-treatment decreases *in vivo* plasma dextrorphan/dextromethorphan ratio and *ex vivo* dextrorphan formation rate by liver microsomes, and increases *in vivo* plasma haloperidol levels and acute haloperidol-induced catalepsy in TG and WT. Thus, we have demonstrated that *in vivo* propranolol given ip acts as an MBI of both hepatic mouse CYP2D and human CYP2D6, and that this inhibition by propranolol is able to affect drug-induced behaviour (catalepsy). See Section 3-2 for detailed results.

4-2-A Long-lasting impact of mechanism-based inhibition

The CYP2D6 MBI paroxetine is known to have long-lasting effects *in vivo* in humans, as a short-term (6 weeks) paroxetine treatment required a 4-week washout period before CYP2D6 disinhibition (Juřica and Žourková, 2013). Longer term (~18 weeks) paroxetine use was reported to need an even longer 6-week washout period, and CYP2D6 inhibition was still present in three of eight patients after 6 weeks (Juřica and Žourková, 2013). Co-medication with propranolol increased drug concentrations of other CYP2D6 substrates (Greendyke and Kanter, 1987; Kiss et al., 2020; Silver et al., 1986) and increased adverse events (Drake and Gordon, 1994; Zhou et al., 1990). These results taken together suggest that patients taking propranolol with other CYP2D6 substrates, especially when those substrates are CYP2D6 MBIs themselves

(e.g.methylenedioxymethamphetamine, paroxetine, cimetidine), are at greater risk for adverse events. Here, we derived inactivation parameters of CYP2D6 mechanism-based inhibition by *in vitro* propranolol in human liver microsomes. These data can be useful in improving our understanding of the clinical effects of CYP2D6 inhibition by propranolol. Careful consideration of treatment with CYP2D6 substrates may be required in patients taking propranolol, even if propranolol has been discontinued prior to the administration of the CYP2D6 substrate.

4-2-B *In vitro* and *in vivo* propranolol inhibition of CYP2D in TG and WT

Propranolol acts as an MBI against mouse CYP2D in TG and WT both *in vitro* (Figures 20 and 21) and *in vivo* (i.e. when propranolol is given ip, Figure 23). The *in vitro* propranolol IC_{50} plots with WT liver microsomes suggest one-site inhibition (Figure 20c), while the *in vitro* propranolol IC_{50} plots with TG liver microsomes suggest two-site inhibition (Figure 20d). These two sites in TG liver microsomes may correspond to mouse CYP2D and human CYP2D6, but it is difficult to distinguish between the two as the dextrorphan formation K_m of mouse CYP2D (Felmlee et al., 2008) and human CYP2D6 (Kerry et al., 1994) overlap. The higher affinity site in TG has a similar IC_{50} value to WT, suggesting that this higher affinity site represents the mouse CYP2D (Figure 20). The lower affinity site in TG liver microsomes (Figure 20) has a similar IC_{50} value to human liver microsomes (Figure 22), and similar to the IC_{50} value previously observed (6.6 μM) (Obach et al., 2006), suggesting that the lower affinity site represents human CYP2D6 (Figure 22). The mouse *Cyp2d* cluster is composed of nine genes (*Cyp2d9, d10, 2d11, 2d12, 2d13, 2d22, 2d26, 2d34, 2d40*) (Nelson et al., 2004) and the mRNA of six are transcribed to different extents in the liver (*Cyp2d9, 2d10, 2d11, 2d22, 2d26, 2d40*) (Renaud et al., 2011). Other organs in WT mice also express CYP2D isozymes (Miksys et al., 2005) and this variation in organ-specific expression is even greater in TG with the addition of differential expression of human CYP2D6 (Cheng et al.,

2013). Inhibition of CYP2D by *in vivo* propranolol given ip may be useful in studying the role of CYP2D and CYP2D6 metabolism within other organs, such as intestine, heart, and brain.

4-2-C Dilution considerations for *in vitro* propranolol inhibition

In this study, we used a two-step method (without dilution) tested at low liver microsome and inhibitor concentrations to determine if *in vitro* propranolol was an MBI of mouse CYP2D and human CYP2D6. This two-step method has the advantage of using a lower liver microsome concentration to reduce inhibitor binding to microsomes, as well as to reduce reactive-intermediate formation in the primary incubation that can affect the secondary incubation (Parkinson et al., 2011). The disadvantage of this method is that without a dilution step, there is an increased chance of inactivation and reversible inhibition occurring in the secondary incubation with the probe substrate, thereby decreasing sensitivity (Grimm et al., 2009). Another approach to determine mechanism-based inhibition is to start with high liver microsome and inhibitor concentrations and then to dilute them 10-fold after a primary preincubation step, prior to the addition of the probe substrate to test for CYP activity in a secondary incubation (Grimm et al., 2009). As described earlier, the dilution step reduces the degree of inactivation and reversible inhibition and should result in increased sensitivity. However, concerns have been raised about this dilution step, given that higher initial liver microsome concentrations can lead to higher amounts of reactive-intermediates formed in the primary incubation which can potentially inflate the extent of protein inactivation that can occur in the secondary incubation (Parkinson et al., 2011). Thus, in this study, we used the former approach.

4-2-D Utility of comparing results between TG and WT

Our data showing that TG have 3-fold higher hepatic V_{max} and turnover than WT (Figure 19) suggests that TG could be used to represent CYP2D6 UMs, while WT could model CYP2D6 EMs, as previously suggested (Cheng et al., 2013; Corchero et al., 2001). A similar fold-difference in activity was observed by Kiss et al. (2018) in human liver microsomes where the rate of dextrorphan formation from dextromethorphan in UM was ~2.5-fold higher compared to EMs. For a number of drugs, high drug levels *in vivo* in *CYP2D6* PMs, as a result of genetic status (Brockmöller et al., 2002; Llerena et al., 2004), or from CYP2D6 MBIs (Juřica and Žourková, 2013; O'Mathúna et al., 2008), could increase the occurrence of adverse events. Using the long-lasting, mechanism-based inhibition by *in vivo* propranolol given ip of mouse CYP2D and human CYP2D6 in TG liver, and of mouse CYP2D in WT liver, we can model the reduction of CYP2D6 UMs and EMs metabolism from an acute exposure to a CYP2D6 MBI. *In vivo* 24-hour ip propranolol pre-treatment was sufficient to alter *in vivo* liver dextrorphan formation from dextromethorphan (as seen with decreased plasma dextrorphan/dextromethorphan ratio) (Figures 23a and 23b); the decrease in plasma dextrorphan/dextromethorphan ratio of saline pre-treated TG (0.43), compared to propranolol pre-treated TG (0.31) resulted in a ratio similar to saline pre-treated WT (0.33). *In vivo* 24-hour ip propranolol pre-treatment was also sufficient to affect *in vivo* haloperidol metabolism (as seen in the modest increase in plasma haloperidol AUC) (Figures 24c and 24d) and behaviour (as seen with the significant increase in haloperidol-induced catalepsy) (Figures 24e and 24f), and was sufficient to show clear proof of MBI inactivation (as seen with the *ex vivo* inhibition of dextrorphan formation rate in liver microsomes from these mice) (Figures 23c and 23d). This was observed despite this *in vivo* ip propranolol pre-treatment being given 24-hour prior to testing. This *in vivo* 24-hour ip propranolol pre-treatment did not affect baseline catalepsy response, suggesting that haloperidol-induced catalepsy was increased due to irreversible

inhibition of CYP2D-mediated haloperidol metabolism and not through the effects of propranolol itself. If even under these circumstances we see a modest shift in *in vivo* metabolism and response in ip propranolol pre-treated mice, then chronic or concurrent propranolol treatments in patients may have a substantial effect on their overall CYP2D6 metabolism. For example, propranolol inhibited the *in vivo* metabolism of another CYP2D6 substrate (debrisoquine) in humans taking propranolol for a week (Rowland et al., 1994). Using both the relative rate of metabolism between the WT and TG, and using the mechanism-based inhibition of CYP2D via *in vivo* propranolol given ip on *in vivo* drug metabolism (e.g. changes in plasma drug levels) and drug response, (e.g. side effects as we have done here using haloperidol-induced catalepsy), this approach could also be useful for investigating novel CYP2D substrates. This approach of giving an MBI such as *in vivo* propranolol ip 24-hour prior to testing is also useful for avoiding the possible confounding pharmacodynamic impacts that competitive inhibitors have *in vivo*, which can hinder their usefulness in modeling decreases in activity. The combined use of *in vivo* ip propranolol and TG mice could also be used to investigate drug interactions, therapeutic effects, and risk for drug dependence and abuse.

In conclusion, our work models the impact of acute *in vivo* ip propranolol exposure (mechanism-based inhibition of CYP2D6) on resulting *in vivo* plasma drug levels and response. Our data also provide evidence for long-lasting inhibition of CYP2D in the liver by *in vivo* propranolol given ip, suggesting caution when prescribing propranolol with other CYP2D6 substrates.

4-3 Study 3: Human CYP2D6 is functional in brain *in vivo*: evidence from humanized CYP2D6-transgenic mice

Herein we examined the role of human CYP2D6 expressed in mouse brain and determined that it was 1) irreversibly inhibited by *in vivo* propranolol given icv, while mouse CYP2Ds in brain were not, and 2) functional both *in vivo* and *ex vivo*, at brain activity levels sufficient to change brain drug concentrations and drug response. This is the first demonstration of human CYP2D6 in brain contributing meaningfully to drug metabolism and response *in vivo*. See Section 3-3 for detailed results.

4-3-A Differences in impact of icv propranolol on TG versus WT

In vivo propranolol given ip is an MBI of mouse CYP2D and human CYP2D6 in the liver (Figures 20, 21, and 22), and thus propranolol was expected to act as an MBI of CYP2D in the brain when given icv. The 4-hour pre-treatment with icv propranolol was initially chosen for two reasons. First, propranolol should be cleared, as 4 hours is greater than 5 half-lives of propranolol (Ito, 2011), reported as ~45 minutes in Swiss Webster mice (Levy et al., 1976). Second, there should be minimal new mouse CYP2D protein synthesis in brain following MBI treatment (while unknown for mouse CYP2D, the human CYP2D6 turnover rate is estimated at ~51-hours) (Venkatakrishnan and Obach, 2005; Yang et al., 2008). However, after *in vivo* 4-hour pre-treatment with icv propranolol, the *in vivo* brain dextrorphan/dextromethorphan ratio was decreased (Figure 26), suggesting there is inhibition of CYP2D in brain *in vivo*. This is consistent with a reported extended propranolol half-life of ~2-hours in mouse brain (Levy et al., 1976). In addition, after an *in vivo* 4-hour pre-treatment with icv propranolol, there was no reduction in *ex vivo* brain DOR formation rate by WT washed brain membranes (propranolol is not present in the *ex vivo* incubations). This suggests that *in vivo* propranolol, when given icv, is a competitive

inhibitor, not an MBI, of mouse CYP2D in brain. In contrast, after an *in vivo* 4-hour pre-treatment with icv propranolol, there was a reduction in *ex vivo* brain DOR formation rate by TG washed brain membranes. This suggests that *in vivo* propranolol, when given icv, is an MBI of human CYP2D6 in TG brain. Consistent with this, after an *in vivo* 24-hour pre-treatment with icv propranolol, there was no impact in WT on mouse CYP2D activity in brain *in vivo* or *ex vivo* (see WT, Figure 26), whereas *in vivo* icv propranolol inhibited human CYP2D6 in TG brain *in vivo* and *ex vivo* (see TG, Figure 26). With both pre-treatment times, in both mouse lines, and for both dextromethorphan and haloperidol, *in vivo* icv propranolol had no effect on *in vivo* plasma drug levels or *ex vivo* hepatic activity, indicating that although *in vivo* propranolol given ip is an MBI for hepatic mouse and human CYP2Ds (see Figure 23), *in vivo* propranolol given icv did not leave the brain in sufficient amounts to alter hepatic activity.

4-3-B Comparison between *in vivo* 24-hour pre-treatment with icv propranolol in TG and WT mice versus rats

As previously described, catalepsy is an animal model used to identify antipsychotic drugs that may produce acute extrapyramidal side effects (Hoffman and Donovan, 1995; Ionov and Severtsev, 2012). In rats, a CYP2D-mediated haloperidol metabolite formed in the brain contributes to haloperidol-induced catalepsy (see results from Figures 5 and 6, and discussion from Section 4-1). Here we demonstrated that catalepsy was more severe in TG than in WT. This is consistent with the additional activity of CYP2D6 in TG brain contributing to more rapid central metabolism of haloperidol and hence greater catalepsy. *In vivo* 24-hour pre-treatment with icv propranolol had no effect on baseline catalepsy response, or on plasma haloperidol levels in both TG and WT (Figures 32 and 33). This is consistent with the very small amount of icv propranolol injected being insufficient to cause a pharmacodynamic or peripheral

pharmacokinetic effects and/or being fully cleared within 24 hours. The *in vivo* 24-hour pre-treatment with icv propranolol before haloperidol administration reduced catalepsy in TG, but not in WT (Figure 31). This provides additional evidence that CYP2D6 in TG brain has sufficient activity to affect drug-response, and that this model can be used to assess the role of CYP2D6 in brain metabolism of other drugs and toxins. Of note, many other antipsychotics are also CYP2D6 substrates (e.g., aripiprazole, risperidone) (Zanger et al., 2004) and thus may be similarly affected by CYP2D6 in the brain.

4-3-C Comparison between *in vivo* 24-hour pre-treatment with icv versus ip propranolol in TG and WT

We showed in Section 3-2 that propranolol given as an *in vivo* ip 24-hour pre-treatment acted as an MBI of mouse liver CYP2D in TG and WT, increased plasma haloperidol, and prolonged catalepsy (a shift in apparent dose as seen in Figure 30b). Some *in vivo* propranolol given systemically may have escaped hepatic metabolism and could have crossed the blood brain barrier, potentially inhibiting local activation by CYP2D in the brain. However, on balance, *in vivo* ip propranolol at this dose and timing resulted in an increase, not a decrease, in catalepsy suggesting a predominant effect of inhibiting the hepatic CYP2D enzyme was on increasing the apparent dose.

4-3-D Mechanism-based inhibitors *in vitro*, but not *in vivo*

While predicting the magnitude of drug-drug interactions is an expected outcome from extrapolating inhibition of drug metabolizing enzymes (e.g. CYPs) *in vitro* with inhibitor concentrations *in vivo* and other relevant information (e.g. dose, protein binding in plasma) (Neal et al., 2003; Yao and Levy, 2002), in practice this is often not the case (Obach et al., 2006). For

example, the inhibitor rofecoxib is a moderate inhibitor of CYP1A2 *in vitro*, but a potent MBI of CYP1A2 *in vivo* (Karjalainen et al., 2006), as demonstrated by a mean 14-fold increase in plasma tizanidine (a CYP1A2 probe substrate) $AUC_{0-\infty}$ in subjects taking rofecoxib daily (Backman et al., 2006). In contrast, the CYP1A2 inhibitors celecoxib (Karjalainen et al., 2008) and tolfenamic (Karjalainen et al., 2007) are potent inhibitors *in vitro*, but not *in vivo*. In some cases, it is evident what mechanism results in the difference between *in vitro* and *in vivo* results, but in other cases, it is harder to determine. For example, initial testing conditions *in vitro* did not account for the very high protein binding that tolfenamic has and so in these *in vitro* conditions tolfenamic was a potent inhibitor; however, albumin is present in *in vivo* conditions and so tolfenamic is not a potent CYP1A2 inhibitor *in vivo* due to the tolfenamic binding to albumin (Karjalainen et al., 2007). Other examples include 17 α-ethenylestradiol (Lin et al., 2002) and gestodene (Guengerich, 1990), which are of potent MBIs of CYP3A4 *in vitro*, but not *in vivo* (Zhang et al., 2008). Other possible reasons for differences between *in vitro* and *in vivo* results include transporter-mediated organ (e.g. brain, liver) uptake and impact of organ-specific metabolism (e.g. brain, intestines) (Obach et al., 2006). The mechanism for how propranolol acts as an MBI of mouse CYP2D in WT liver, but not an MBI of mouse CYP2D in WT brain, is yet unknown, and future work may help elucidate this finding.

4-3-E Differences between CYP2D in liver and brain

We have shown that *in vivo* propranolol given ip 24 hours prior to testing acts as an MBI of mouse CYP2D in liver (Figure 23), but that *in vivo* propranolol given icv 4 and 24 hours prior to testing does not act as an MBI of mouse CYP2D in brain (Figures 26 and 28). In addition to the types of reasons outlined above (e.g. drug binding, organ penetration, unknown mechanisms), this may be due to different complements of mouse CYP2D isoforms expressed in liver and brain

and the interaction of the inhibitor with these different enzymes. The mRNA of six CYP2D isozymes (CYP2D9, 2D10, 2D11, 2D22, 2D26, 2D40) were found in C57BL/6J mouse liver, but the mRNA of only two (CYP2D10, 2D22) were found in C57BL/6J mouse brain (Renaud et al., 2011). CYP2D22, but not CYP2D10, was found in mouse brain (Blume et al., 2000; Choudhary et al., 2003), and the mRNA of CYP2D isoforms other than CYP2D10 and CYP2D22 were transcribed in mouse brain (Yamaori et al., 2017); differences between studies in brain isoforms detected may be due to differing mouse strains or techniques used. Interestingly, the cellular and regional expression pattern of mouse brain CYP2D (Miksys et al., 2005) is similar to that of human CYP2D6 in both human (Miksys et al., 2002; Siegle et al., 2001) and TG (Cheng et al., 2013) brain, with some minor differences (see (Miksys et al., 2005) for a detailed comparison). Organ-specific transcription of CYP2D isoforms has also been seen in rats, where CYP2D4 mRNA is predominantly found in brain, but not in liver (Hiroi et al., 1998a; Komori, 1993). In addition, it is not clear which other CYP2D isoforms are translated and functional in brain, relative to liver. Propranolol metabolism by CYP2D *in vitro* is required before CYP2D can be irreversibly inhibited in a mechanism-based manner (Figures 20 and 21). Rat CYP2D1, 2D2, 2D3, and 2D4, and human CYP2D6 can mediate propranolol 4-, 5-, and 7- hydroxylation, and *N*-desisopropylation *in vitro* to different extents (e.g. CYP2D1 catalyzed neither propranolol 5-hydroxylation nor propranolol 7-hydroxylation) (Hiroi et al., 2002). Likewise mouse CYP2D22, which is found in brain, has a differing binding affinity, rate of metabolism, inhibition profile, and allosteric behaviour compared to human CYP2D6 (McLaughlin et al., 2008; Yu and Haining, 2006). It is possible that CYP2D isoforms expressed in mouse brain metabolizes *in vivo* propranolol differently relative to mouse CYP2D isoforms expressed in liver. Thus, our data is consistent with enzymatically active mouse CYP2D isoforms expressed in brain being

insensitive to *in vivo* propranolol given icv 4 and 24 hours prior to testing as an MBI (Figures 26 and 28), while sensitive to it as a competitive inhibitor (Figures 26).

The wide variation in CYP2D6 expression in brain between humans is influenced by genetic variation, and is also associated with age and exposure to inducers (Mann et al., 2008; Mann et al., 2012; Miksys et al., 2002; Miksys and Tyndale, 2004; Miksys and Tyndale, 2013). Variation in CYP2D6 is tissue-specific, whereby human smokers and alcoholics have higher CYP2D6 in brain (Mann et al., 2008; Miksys et al., 2002; Miksys and Tyndale, 2004), but similar liver CYP2D6 (Bock et al., 1994; Funck-Brentano et al., 2005; Steiner et al., 1985), compared to non-smokers and non-alcoholics, respectively. Moreover, chronic nicotine and alcohol exposure in rats and African Green monkeys induces brain, but not liver, CYP2D (Mann et al., 2008; Miller et al., 2014; Yue et al., 2008). The role of variable CYP2D in brain could be explored with this approach of using TG expressing CYP2D6 in brain in combination with an *in vivo* 24-hour pre-treatment with icv propranolol. The role of CYP2D6 in brain drug response including therapeutic effects, abuse liability, and toxicity could be examined for the many classes of CYP2D6 substrates, which include antipsychotics, antidepressants, opioids, and psychostimulants (Zanger et al., 2004; Zanger and Schwab, 2013; Zhou, 2016). Furthermore, as some pharmaceutical and environmental neurotoxins are among CYP2D6 substrates, this model may be useful for examining the role of CYP2D6 in brain in susceptibility to and protection from neurotoxicity and neurodegeneration.

Our current understanding of variation in activity of human CYP2D6 in the brain, in terms of its impact on drug metabolism and response, and how this variation relates to brain function and behaviour, is still lacking. While associations between genetic variation in human *CYP2D6* and personality (Kirchheiner et al., 2006; Peñas-Lledó et al., 2009; Roberts et al., 2004), cognition

(Peñas-Lledó et al., 2009; Stingl et al., 2012), schizophrenia (Llerena et al., 2007), eating disorders (Peñas-Lledó et al., 2012b; Peñas-Lledó et al., 2012c), and suicidality (Peñas-Lledó et al., 2012a; Zackrisson et al., 2010) have been documented, suggesting a role for CYP2D6 in the brain, the mechanisms remain unknown. The transgenic mouse and inhibition approach described here can be used for the first time to directly test the function of human CYP2D6 in the brain, independent of the liver.

In conclusion, the *in vivo* 24-hour pre-treatment with icv propranolol is a new tool to selectively and irreversibly inhibit human CYP2D6 in TG brain, providing a novel approach to examine the impact of variation in CYP2D6 in brain on local drug metabolism and response (using WT as controls where needed). CYP2D6 in brain may influence the severity of acute extrapyramidal side effects from haloperidol via a catalepsy-causing metabolite formed within the brain. Our findings demonstrate that human CYP2D6 in brain is functional and that it can meaningfully alter a central response to a drug given peripherally.

4-4 Variation of CYP2D in the brain

4-4-A Regional variation of CYP2D in the brain

The level of CYP2D expression varies among brains regions; these patterns of expression have substantial overlap in humans, rats and mice (Miksys et al., 2005). For example, there is high cerebellar expression in all three species (Miksys et al., 2005). In humans, CYP2D6 expression is highest in the cerebellum, and the lowest in the substantia nigra (Mann et al., 2012; Miksys et al., 2002). In monkeys, CYP2D expression is highest in the brainstem and the substantia nigra, and lowest in the frontal cortex and the hippocampus (Mann et al., 2008; Miller et al., 2014). Similar to monkeys, there is high CYP2D expression in the cerebellum, the substantia nigra, and the hippocampus in both rats (Miksys et al., 2000) and mice (Miksys et al., 2005), suggesting that lower CYP2D expression in the substantia nigra might be specific to humans. This high expression of CYP2D in the cerebellum is accompanied by high CYP2D enzymatic activity in rats (Tyndale et al., 1999). The results in Section 3-3-A suggest that in mice the cerebellum also has high baseline mouse CYP2D enzymatic activity that is further increased in TG (Figure 25).

In addition to differences in brain-region CYP2D expression, there are also differences in cellular expression between humans, rats, and mice. CYP2D6 is expressed in human neuronal and non-neuronal cells, including pyramidal cells of the cortex and hippocampus, pigmented neurons of the substantia nigra, dopaminergic terminals in the striatum, Purkinje and glial cells of the cerebellum, and astrocytes (Dutheil et al., 2009; Gilham et al., 1997; Miksys et al., 2002; Siegle et al., 2001). The cellular expression of CYP2D in the brains of rats (Miksys et al., 2000) and mice (Miksys et al., 2005) is similar to the cellular expression of CYP2D6 in human brain (Mann et al., 2012; Miksys et al., 2002), with high expression of CYP2D in pyramidal cells of the cortex, neurons and glia of the hippocampus, Purkinje cells of the cerebellum, internal

granular layer of the olfactory bulbs, and glia and neurons in the caudate putamen. In TG, staining specifically for human CYP2D6 demonstrated high expression of CYP2D6 in pyramidal cells of the frontal and parietal cortex, and the cytoplasmic bridges in the striatum, and moderate staining in molecular and granular cell layers of the cerebellum and molecular and pyramidal cell layers of the hippocampus (Cheng et al., 2013). This suggests similar expression of human CYP2D6 in the brains of humans and TG. The relative expression of human CYP2D6 compared to mouse CYP2D in TG mouse brain was not characterized (via immunoblotting), as antibodies that detect mouse CYP2D, but not human CYP2D6, in brain were unavailable.

Immunoblotting for CYP2D in the brains of rats (Miksys et al., 2000), mice (Miksys et al., 2005), and monkeys (Mann et al., 2008; Miller et al., 2014) shows multiple bands, whereas humans only show a single band (CYP2D6) (Mann et al., 2012; Miksys et al., 2002), suggesting that these animals express multiple CYP2D isozymes in the brain. In rats, CYP2D4 mRNA has been detected in the brain (Hiroi et al., 1998a; Komori, 1993; Wyss et al., 1995) and CYP2D4 protein was detected by Western blot (Wyss et al., 1995). There is also expression of CYP2D1 and CYP2D5 mRNA (Miksys et al., 2000) and protein (Riedl et al., 1999) in rat brain. In mice, the mRNA of two isozymes, CYP2D10 and CYP2D22, has been detected in the brain (Renaud et al., 2011). In monkeys, two immunoreactive bands (molecular weights of 52 and 55 kilodaltons (kDa)) for CYP2D have been detected in the brain and one immunoreactive band (co-migrated with the lower 52 kDa band) for CYP2D has been detected in the liver (Mann et al., 2008; Miller et al., 2014). Of these two bands in the brain, the lower band (52 kDa) increases after chronic nicotine induction; the upper band (55 kDa) in the brain, as well as the sole band (52 kDa) in the liver is unchanged (Mann et al., 2008; Miller et al., 2014). This suggests that the lower band in the monkey brain is regulated differently to the upper band in the brain and to the sole band in the liver by nicotine. It is possible that the difference in the impact of *in vivo* 24-hour pre-

treatment with icv propranolol on TG and WT on CYP2D activity in the brain is due to varying substrate specificity and enzymatic activity profiles between CYP2D isozymes, and due to varying regulation of the same isozyme between tissues.

4-4-B Drugs that impact CYP2D in the brain

CYP2D in the brain can be induced by a number of centrally-acting drugs, including nicotine (Yue et al., 2008), antipsychotics (Haduch et al., 2011; Hedlund et al., 1996) and antidepressants (Haduch et al., 2011), in a drug by brain region manner, while CYP2D in the liver is considered uninducible (Edwards et al., 2003; Madan et al., 2003). Hedlund et al. (1996) found that a 24-hour pre-treatment with the antipsychotic clozapine in rats increased CYP2D4 immunoreactivity in Purkinje and granular neurons of the cerebellum, granular neurons of the olfactory bulb, neurons of the substantia nigra, and neurons of the ventral tegmental area, relative to untreated rats that had very little immunoreactive CYP2D4 in rat brain; CYP2D in the liver was not affected by this clozapine pre-treatment. CYP2D mRNA in the cerebellum and olfactory bulbs, areas with induced CYP2D4, was unchanged by clozapine treatment (Hedlund et al., 1996). This suggests that CYP2D induction in the brain from clozapine is not by a transcriptional mechanism. Similarly, there was also no change in CYP2D mRNA levels in the brain after seven day sc nicotine treatment in rats, a treatment that increased CYP2D in the striatum, cerebellum, and hippocampus, without changing levels of CYP2D in the liver (Yue et al., 2008). Haduch et al. (2011) also found that a two-week treatment with the antipsychotic thioridazine increased CYP2D4 levels in in the substantia nigra and the cerebellum. Haloperidol treatment for at least two weeks did not induce CYP2D in the rat brain (Hedlund et al., 1996) or liver (Daniel et al., 2005; Hedlund et al., 1996), suggesting that rats given the chronic haloperidol treatment

described in Section 3-1-D had baseline levels of CYP2D in the brain, without the induction that may have been observed from other antipsychotics like clozapine and thioridazine.

Chronic treatment with drugs can have differential effects on CYP2D activity in the brain and liver. For example, administering to rats for two weeks daily injections of the antidepressants imipramine and mirtazapine decreased and increased CYP2D activity in the liver, respectively (Daniel et al., 2002); the same antidepressant treatments did not change CYP2D activity or CYP2D levels in the brain, compared to saline treated rats (Haduch et al., 2004). A two-week treatment with another antidepressant, fluoxetine, increased CYP2D4 levels and CYP2D activity in rat cerebellum, decreased CYP2D4 levels and CYP2D activity in the nucleus accumbens and striatum (Haduch et al., 2011), and decreased CYP2D activity in the liver (Daniel et al., 2002). A two-week treatment with the antipsychotic thioridazine increased CYP2D activity in the striatum and cerebellum of rats (Haduch et al., 2011), despite this thioridazine treatment resulting in decreased CYP2D activity in the liver (Daniel et al., 2005).

4-4-C Other factors that contribute to variation of CYP2D in the brain

Age and sex are also factors that may impact CYP2D in the brain. In the liver, human CYP2D6 levels rapidly increased after birth and remained stable throughout adulthood (Treluyer et al., 1991). Similarly, CYP2D6 levels in the brain are higher between birth and the first year of age, and remained similar until 20 years of age (Mann et al., 2012). However, unlike liver, from 20 to at least 80 years of age, CYP2D6 levels were even higher in brain regions such as the frontal cortex, the substantia nigra, and the cerebellum (Mann et al., 2012). While this suggests that the increase in human CYP2D6 in the brain during adulthood is a natural part of aging (Hernandez et al., 2011; Thompson et al., 2010) or is a response to age-related changes in the brain (Bartzokis et al., 2003; Fjell and Walhovd, 2010; Piguet et al., 2009), it is also possible that this increase is a

response to increased exposure to drugs, food, or environmental toxins. As discussed previously, chronic exposure to exogenous drugs such as nicotine (Mann et al., 2008; Miller et al., 2014) and alcohol (Miller et al., 2014) induced CYP2D in the brains of monkeys, and clinical drugs such as clozapine (Hedlund et al., 1996), thioridazine (Haduch et al., 2011), and nicotine (Yue et al., 2008) induced CYP2D in the brains of rats, without affecting CYP2D levels in the liver.

Another possibility is that CYP2D6 levels in the brain are affected by sex. Not much is known about the impact of sex on CYP2D6 in the brain, but animal studies suggest that sex hormones may play a role in sex-specific regulation of CYP2D in the brain. Ovariectomized rats had decreased CYP2D mRNA levels in the brain compared to non-ovariectomized rats, suggesting that estrogen (as an example) may have reduced CYP2D expression in the brain (Bergh and Strobel, 1996). Seven day sc treatment of testosterone in ovariectomized rats greatly increased CYP2D mRNA levels in the brain; however, seven day sc treatment of both testosterone and estrogen in ovariectomized rats decreased CYP2D mRNA levels in the brain compared to ovariectomized rats given testosterone alone (Bergh and Strobel, 1996). This suggests that testosterone may induce CYP2D expression in the brain, and that estrogen may reduce testosterone's stimulatory effect on CYP2D levels in the brain. This was supported by the work of Baum and Strobel (1997) where a similar seven day ip treatment of testosterone increased CYP2D mRNA levels in the brain of non-ovariectomized female rats. Further, seven day ip progesterone treatment decreased CYP2D mRNA levels in the brain of non-ovariectomized female rats (Baum and Strobel, 1997). This suggests that progesterone, like estrogen, may reduce CYP2D expression in the brain. Female rats and mice, and the impact of estrous cycle, were not investigated in the studies in this thesis but may be an interesting research avenue for the future.

4-5 Clinical relevance of CYP2D in brain

4-5-A Potential impact of CYP2D in brain on therapeutic drug use

CYP2D6 is involved in the metabolism of 20-30% of all clinically used drugs (Yu et al., 2004; Zanger and Schwab, 2013), a number of which are drugs that act on the brain, such as antidepressants, antipsychotics, and opioids (Table 3) (Zhou, 2016). CYP2D in rat (Chen et al., 1990; Coleman et al., 2000; Lin et al., 1992; McMillan and Tyndale, 2015; Voirol et al., 2000) and dog (Tyndale et al., 1991) brain are functional *ex vivo* and able to metabolize a number of centrally acting substrates. Although inter-individual variation in human drug metabolism is primarily determined by *CYP2D6* genetic variation, CYP2D6 activity can still greatly differ between individuals, even within the same *CYP2D6* genotype (Gaedigk, 2013; Ingelman-Sundberg et al., 2007). Despite the large role that the liver plays in metabolizing drugs, not all people respond to centrally-acting drugs even when plasma drug or metabolite levels (e.g. antipsychotics) are optimal (Michels and Marzuk, 1993). Local brain drug metabolism of centrally-acting drugs may play a role in determining response. Simulations have shown that localized CYP2D6 in specific brain-regions may impact steady-state drug levels in the brain, without changing plasma drug levels (Britto and Wedlund, 1992). This suggests that differences in expression of CYP2D6 in the brain may impact metabolism by CYP2D6 within the brain and could therefore change drug response.

Inter-individual CYP2D6 variation may contribute to differences in drug effects, including non-standard or adverse drug responses from a standard dose (Gaedigk, 2013; Wang et al., 2009; Zanger et al., 2008). Antipsychotics, such as risperidone and haloperidol, are metabolized by CYP2D6 (Zhou, 2016). However, as discussed previously, plasma levels are not always a good indicator of drug response even when the optimal dose of drug is given (Michels and Marzuk,

1993). For example, plasma levels of the risperidone active moiety (i.e. the combination of risperidone and 9-hydroxyrisperidone levels) do not correlate with drug response in patients with schizophrenia (Lane et al., 2000; Riedel et al., 2005; Spina et al., 2001). Patients taking these antipsychotics can also develop adverse drug responses such as extrapyramidal side effects that include parkinsonian tremors, stiffness, dystonia and tardive dyskinesia (Shirzadi and Ghaemi, 2006). Plasma levels of risperidone active moiety also do not correlate with extrapyramidal symptoms in patients with schizophrenia (Riedel et al., 2005). This suggests that drug metabolism in the brain by CYPs, such as CYP2D6, may influence both drug response and the appearance of adverse events.

CYP2D6 PMs have no detectable CYP2D6 in the liver (Mann et al., 2012; Zanger and Schwab, 2013) or the brain (Mann et al., 2012). Therefore, *CYP2D6* PMs are unable to metabolize antipsychotics in the liver or in the brain, which may contribute to the association between increased extrapyramidal symptoms and *CYP2D6* PM genotype (Brockmöller et al., 2002; Schillevoort et al., 2002; Tamminga et al., 2003). However, extrapyramidal symptoms are also experienced by *CYP2D6* EMs. Given that *CYP2D6* EMs have wide variation in CYP2D6 expression in the brain (Mann et al., 2012), it is possible that lower CYP2D6 expression in the brain of *CYP2D6* EMs may contribute to reduced inactivation of antipsychotics and result in increased extrapyramidal symptoms. Smokers have increased CYP2D6 in the brain, including in the basal ganglia (Mann et al., 2008; Miksys and Tyndale, 2004), without any changes to CYP2D6 in the liver (Bock et al., 1994; Funck-Brentano et al., 2005; Steiner et al., 1985). The impairment of dopamine release from the basal ganglia is suspected to be a cause of extrapyramidal side effects from antipsychotics (Jabs et al., 2003; Kapur et al., 2000). Smokers, versus non-smokers, have a lower prevalence for antipsychotic-induced parkinsonism and score lower on extrapyramidal symptoms scales (Jabs et al., 2003). The decrease in extrapyramidal

symptoms in smokers might be due to higher CYP2D6 in their brains allowing for greater inactivation of antipsychotics. Therefore, variation in brain-specific CYP2D6 may impact both drug response, and adverse events from antipsychotics.

In humans, the antipsychotic haloperidol has been shown to improve schizophrenia symptoms such as aggressiveness, delusions, hallucinations, impulsiveness and states of excitement (Adams et al., 2013). Studying these behaviours in animals can be challenging so instead we study animal behavioural correlates. Haloperidol reverses behavioural correlates of positive symptoms, making animal models that use haloperidol as a positive control useful in studying these symptoms of schizophrenia (Jones et al., 2011). The pharmacokinetics of haloperidol differ between rats, mice, and humans, with plasma half-lives of 1.5 hours (Cheng and Paalzow, 1992), 0.75 hours (Levy et al., 1976), and >15 hours (Huang et al., 1996), respectively. As shown in Figure 4, the acute side-effects of haloperidol, modeled here by catalepsy, occurred within 15 minutes after haloperidol exposure and the maximal effect was attained within 30 minutes and was sustained for at least 120 minutes. From 30 minutes on, sustained catalepsy was unrelated to decreasing brain and plasma haloperidol levels. This suggests an initial effect of haloperidol dose, which in this model becomes rapidly disassociated from haloperidol levels and half-life. Thus, while human and rat half-lives differ substantially, we do not anticipate that this affects interpretation of the acute catalepsy responses illustrated here. To model tardive dyskinesia resulting from chronic haloperidol exposure, rats were given haloperidol via a continuous infusion. This mirrors haloperidol pharmacokinetics in humans, resulting in sustained rat plasma levels of 12 ng/ml, consistent with those observed in humans (i.e. 11 ng/ml) (Panagiotidis et al., 2007), suggesting similar brain exposure. Additional behaviours affected by haloperidol administration, such as locomotion, attention, and prepulse inhibition of acoustic startle (Jones et al., 2011), were not investigated in this thesis and could be investigated in future studies to

understand the impact of manipulating human CYP2D6 in the brain on other behaviours affected by antipsychotic use.

The metabolism of the opioids codeine and oxycodone in the brain and the accompanying analgesic response *in vivo* was altered by the manipulation of CYP2D in the brain (McMillan et al., 2019; McMillan and Tyndale, 2015). Brain morphine levels were higher and peak analgesia was earlier in rats given ip codeine, which was metabolized by CYP2D into morphine (Dayer et al., 1988), compared to rats given ip morphine, despite both sets of rats having similar peak plasma morphine levels (McMillan and Tyndale, 2015). The lower brain morphine levels and later peak analgesia in rats given ip morphine, relative to ip codeine, suggests that peripheral morphine has difficulty crossing the blood brain barrier (Chen et al., 1990); compared to codeine, morphine is less permeable across the blood brain barrier (Oldendorf et al., 1972) and is actively effluxed out of the brain (Bouw et al., 2000; Xie and Hammarlund-Udenaes, 1998). Therefore, the higher brain morphine levels in rats given ip codeine suggests that codeine is metabolized into morphine in the brain by CYP2D, resulting in the earlier peak analgesia. Brain morphine levels and resulting analgesia following codeine were lower in rats after brain-specific inhibition of CYP2D via *in vivo* 24-hour icv pre-treatment with propranolol; in contrast, brain morphine levels and resulting analgesia following codeine were higher in rats after brain-specific induction of CYP2D via daily *in vivo* sc treatment with nicotine for seven days (McMillan and Tyndale, 2015). The effect of CYP2D induction on brain morphine levels and analgesia following codeine is reversed when CYP2D in the brain is inhibited (McMillan and Tyndale, 2015). Together this suggests that differences in brain morphine levels and analgesia, following codeine, are due to differences in CYP2D activity in the brain.

Oxycodone is metabolized by brain CYP2D into oxymorphone (Klimas et al., 2013). Unlike codeine, where its metabolite morphine is responsible for analgesia, the parent oxycodone, not the metabolite oxymorphone, is responsible for analgesia (McMillan et al., 2019). Brain oxycodone levels and resulting analgesia are higher in rats after brain-specific inhibition of CYP2D; in contrast, brain oxymorphone levels are higher but resulting analgesia is lower in rats after brain-specific induction of CYP2D (McMillan et al., 2019). The effect of CYP2D induction in the brain on analgesia is also reversed when CYP2D in the brain is inhibited (McMillan et al., 2019). Inhibiting and inducing CYP2D in the brain had no effect on analgesia following morphine (McMillan and Tyndale, 2015) or oxymorphone (McMillan et al., 2019); these metabolites of codeine and oxycodone, morphine and oxymorphone respectively, were not metabolized by CYP2D. This suggests that it is the brain-specific changes to CYP2D activity that result in changes to brain drug levels of codeine vs morphine and oxycodone vs oxymorphone, and resulting analgesia. It is possible that brain-specific changes to CYP2D6 in human brain may also impact drug response (e.g. analgesia) from opioids, with the direction of these impacts depending on whether the parent opioid or CYP2D6 metabolite is responsible for the relevant drug response.

The model we describe in Sections 4-3-A and 4-3-B allows for the specific *in vivo* inhibition of human CYP2D6 in TG brain, without inhibiting mouse CYP2Ds in TG brain, and without inhibiting human CYP2D6 or mouse CYP2Ds in TG liver. This model can also utilize WT mice to act as negative controls for the impact of *in vivo* 24-hour pre-treatment with icv propranolol. Future experiments can utilize this model to study emerging compounds that are potential CYP2D6 substrates. For example, to investigate the impact of inter-individual variation in CYP2D6 in human brain on drug metabolism and response (i.e. therapeutic effect and possible side effects). However, it is important to remember that this is a model system using exogenously

expressed human CYP2D6 in TG. Thus, we have demonstrated that CYP2D6 expressed in the mouse brain can have a role in drug response *in vivo.* Despite the similarity in CYP2D6 expression patterns (e.g., in neurons of the frontal cortex, the striatum, and the molecular and granular layer of the cerebellum) in human (Miksys et al., 2002; Siegle et al., 2001) and TG (Cheng et al., 2013) brains, these data do not necessarily directly reflect expression or function of endogenous CYP2D6 in human brain. However, this TG model provides an excellent first step towards understanding the impact the human CYP2D6 may have in the brain.

4-5-B Impact of CYP2D in brain on endogenous metabolism and risk for psychiatric disorders

CYP2D6 is expressed in specific regions of the brain where it may be involved in the synthesis and metabolism of endogenous compounds that act on the brain (Funae et al., 2003; Miksys and Tyndale, 2013). These endogenous compounds include monoamine-like substrates such as dopamine (Hiroi et al., 1998b; Miller et al., 2001; Niwa et al., 2008) and epinephrine (Hiroi et al., 1998b) (Table 4). CYP2D6 can catalyze 5-methoxytryptamine *O*-demethylation to serotonin 1) *in vitro* using recombinant human CYP2D6 (Yu et al., 2003a), and 2) using human liver microsomes (Haduch et al., 2013). CYP2D6 transgenic mice were similarly able to catalyze 5-methoxytryptamine to form serotonin *in vivo*; liver microsomes from CYP2D6 transgenic mice formed serotonin 16-fold higher compared to liver microsomes from WT *in vitro* (Yu et al., 2003a). This may partially explain why serotonin, and its metabolite 5-hydroxyindoleactic acid, levels are higher in TG compared to WT brain, given that TG express both human CYP2D6 and mouse CYP2D in the brain (Cheng et al., 2013). These results suggest that human CYP2D6, specifically CYP2D6 in the brain, may play an endogenous role in modulating neurotransmitters.

Table 4: Examples of endogenous CYP2D6 substrates in the brain

	Substrate	Product	References
Amine/Neurotransmitters	3-Methoxyphenethylamine	*m*-Tyramine	Miller et al. (2001)
	4-Methoxyphenethylamine	*p*-Tyramine	Miller et al. (2001)
	5-Methoxytryptamine	Serotonin	Yu et al. (Yu et al., 2003a)
	p-Octopamine	Norepinephrine	Hiroi et al. (Hiroi et al., 1998b)
	p-Synephrine	Epinephrine	Hiroi et al. (Hiroi et al., 1998b)
	p/m-Tyramine	Dopamine	Hiroi et al. (Hiroi et al., 1998b), Miller et al. (2001), Niwa et al. (2008)
	Harmaline	Harmalol	Yu et al. (2003b)
	Harmine	Harmol	Yu et al. (2003b)
	Substrate	Reaction	References
Steroids	Allopregnanolone	21-hydroxylation	Kishimoto et al. (2004)
	Estradiol	2-hydoxylation	Lee et al. (2003)
	Estrone	2-hydoxylation	Lee et al. (2003)
	Progesterone	2β-, 6β-, 16α- or 21-hydroxylation	Hiroi et al. (2001), Kishimoto et al. (2004)
	Testosterone	2β-, 6β-, or 17α-hydroxylation	Hiroi et al. (2001)

Adapted from Zhou 2016.

CYP2D6 can also metabolize neural substrates that are active in the brain, including steroids such as progesterone (Hiroi et al., 2001; Kishimoto et al., 2004) and testosterone (Hiroi et al., 2001), and fatty acids such as anandamide (Snider et al., 2008). Progesterone treatment in

women (Hlatky et al., 2002) and testosterone treatment in men alters mood (Kouri et al., 1995; Pope et al., 2000). Anandamide is an endocannabinoid implicated in the regulation of mood and anxiety, emotional processing, and associated to depression pathophysiology (Bambico and Gobbi, 2008). Variation in CYP2D6 expression, for instance from chronic exposure to nicotine or alcohol, may influence neural substrates levels and therefore affect mood.

In humans, genetic variation in *CYP2D6* is associated with differing personality traits (Kirchheiner et al., 2006; Peñas-Lledó et al., 2009; Roberts et al., 2004) and with differing risks for psychopathology (Brockmöller et al., 2002; Dahl et al., 1998; Llerena et al., 2007; Peñas-Lledó et al., 2012b; Peñas-Lledó et al., 2012c). *CYP2D6* PMs have been associated with greater impulsivity (Peñas-Lledó et al., 2009) and heightened anxiety versus *CYP2D6* EMs (González et al., 2008; Llerena et al., 1993). Moreover, *CYP2D6* genotype has been associated with differences in resting brain perfusion rates (Kirchheiner et al., 2011), brain activity in areas related to alertness during a cognitive task (Stingl et al., 2012), and neurocognition as assessed with a systematic battery of cognitive tests (Peñas-Lledó et al., 2009), suggesting a role for CYP2D6 in the brain. *CYP2D6* genotype has also been associated with schizophrenia, as schizophrenic inpatients were less likely to be *CYP2D6* PMs than *CYP2D6* EMs (Brockmöller et al., 2002; Dahl et al., 1998; Llerena et al., 2007). There is a higher frequency of eating disorders in patients genotyped as *CYP2D6* UMs (Peñas-Lledó et al., 2012b) and in patients phenotyped as CYP2D6 UMs by debrisoquine hydroxylation (Peñas-Lledó et al., 2012c). German patients with depression that are genotyped as *CYP2D6* UMs have an increased risk of suicidality compared to other *CYP2D6* genotypes (Stingl and Viviani, 2011). In a study by Zackrisson et al. (2010) looking at individuals who died of fatal intoxication (n=239), suicide (n=254), or natural death (n=205), they found that there was a higher proportion of individuals genotyped as *CYP2D6* UMs who died from suicide (n=12) and who died from intoxication (n=6) compared to

individual genotyped as *CYP2D6* UMs who died from natural causes (n=1). Lastly, *CYP2D6* UMs who attempt suicide do so with greater severity (i.e. more active planning or preparation of the suicide attempt) (Peñas-Lledó et al., 2012a) and exhibit greater personality psychopathology (e.g. avoidant, borderline, histrionic, paranoid) compared to other *CYP2D6* genotypes (Blasco-Fontecilla et al., 2014).

Given the large number of neural substrates that are metabolized by CYP2D6 to neurotransmitters, the expression of CYP2D6 in the brain may play a subtle modulatory role in brain homeostasis. Therefore, altered CYP2D6 activity in the brain may affect the endogenous metabolism of neural substrates and may result in changes to mood, personality traits, and risk of developing psychiatric disorders (Miksys and Tyndale, 2013).

4-5-C Impact of CYP2D in brain on risk for neurotoxicity

Being a *CYP2D6* PM has been associated with increased risk for Parkinson's disease (Anwarullah et al., 2017; Lu et al., 2013; McCann et al., 1997) , and this risk for *CYP2D6* PMs increased with exposure to neurotoxic pesticides (Deng et al., 2004; Elbaz et al., 2004). The pro-neurotoxin MPTP can readily enter the brain, producing dopaminergic neuronal loss that resembles the neurodegeneration seen in patients with Parkinson's disease (Przedborski, 2007). MPTP and other pro-neurotoxins can be detoxified by CYP2D6 (Coleman et al., 1996), which is expressed in brain regions affected by Parkinson's disease such as the caudate and the substantia nigra (Mann et al., 2012; Miksys et al., 2002). CYP2D6, and many other CYPs, are expressed in subcellular compartments, including the mitochondria in human brain (Bhagwat et al., 2000; Dutheil et al., 2009) and liver (Sangar et al., 2009) . Since the mitochondria are commonly damaged by a number of neurotoxins implicated in Parkinson's disease (Burbulla et al., 2010), CYP2D6 metabolism in the mitochondria could play a role in neuroprotection.

Human smokers have an approximately 50% lower relative risk of developing Parkinson's disease compared to age-matched non-smokers (Alves et al., 2004). Chronic nicotine administration induces CYP2D in brain (but not liver) in rats and African Green monkeys (Mann et al., 2008; Miller et al., 2014; Yue et al., 2008). This suggests that nicotine may be neuroprotective by increasing CYP2D-mediated neurotoxin inactivation in brain. Based on post-mortem studies, individuals with Parkinson's disease were more likely to have the *CYP2D6*4* null allele (associated with no detectable CYP2D6 in liver) (Mann et al., 2012; Zanger and Schwab, 2013) than individuals without Parkinson's disease (Mann et al., 2012). In addition, individuals with Parkinson's disease had 50% lower CYP2D6 levels in brain compared to age-matched individuals without Parkinson's disease, even after controlling for *CYP2D6* genotype (Mann et al., 2012). Taken together, this suggests that CYP2D6 in brain may detoxify neurotoxins, reducing the risk for these disorders. In contrast, individuals with lower CYP2D6 expression in brain, due to genotype or environmental exposures, may be at increased risk for Parkinson's disease. Evidence for a functional role of human CYP2D6 in brain in *in vivo* neurotoxicity and/or neuroprotection could be further assessed using this model of TG mice and *in vivo* 24-hour icv propranolol pre-treatment.

ß-Carbolines show structural similarities to MPP^+ (Matsubara et al., 1993) (Figure 34), the neurotoxic metabolite of MPTP that causes a parkinsonian syndrome in humans and animals (Langston et al., 1983). In addition to structural similarities, ß-carbolines and MPP^+ both produce neurotoxicity, including in mice the induction of bradykinesia, the reduction of striatal dopamine, and the reduction of tyrosine hydroxylase containing cells (Matsubara et al., 1998), and in rats the reduction of striatal dopamine (Neafsey et al., 1995; Neafsey et al., 1989). ß-Carbolines are naturally occurring tryptophan derivatives that are detected in plants and animals (Herraiz and Galisteo, 2014), including the human brain (Airaksinen and Kari, 1981). Blood levels of ß-

carbolines correlate with neurodegenerative measures (Louis et al., 2007) and cerebrospinal fluid levels of ß-carbolines are elevated in Parkinson's patients (Kuhn et al., 1996). Many ß-carbolines are inactivated by CYP2D (Herraiz et al., 2006; Herraiz et al., 2008; Yu et al., 2003b); for example, harmine is metabolized by CYP2D6 to the inactive metabolite, harmol (Yu et al., 2003b). Harmine is a ß-carboline that can induce hypothermia (Abdel-Fattah et al., 1995) and tremor (Kelly and Naylor, 1974); harmine is mainly *O*-demethylated by CYP2D6, and partially by CYP1A1, CYP1A2, CYP2C9, and CYP2C19 (Yu et al., 2003b). Given the involvement of CYP2D6 in metabolizing ß-carbolines, it is possible that CYP2D6 may protect against ß-carboline induced neurotoxicity.

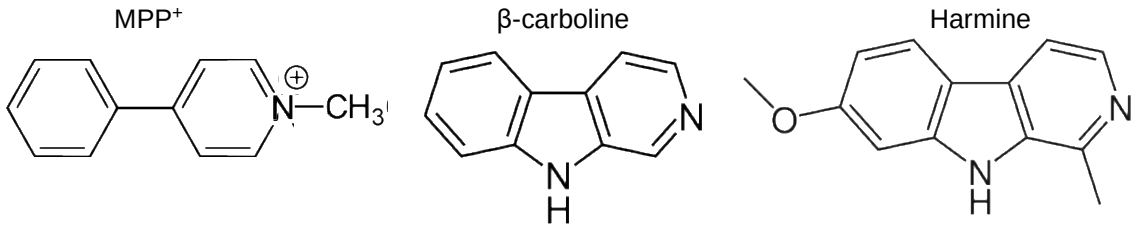


Figure 34. MPP+ (1-methyl-4-phenylpyridinium) and general ß-carbolines have similar structures. *Harmine is an example of a ß-carboline.*

We have used our model employing TG mice and *in vivo* 4-and 24-hour icv propranolol pre-treatments to investigate the role of human CYP2D6 in brain in neuroprotection against harmine (see Appendix A for this manuscript). Harmine given peripherally caused hypothermia and tremor in TG and WT mice. Following *in vivo* 4-hour pre-treatment with icv propranolol tremor and hypothermia were exacerbated in TG, and hypothermia was in WT. Further, harmine-induced hypothermia was exacerbated in TG by *in vivo* 24-hour pre-treatment with icv propranolol, having no effect in WT mice. As shown for experiments with haloperidol given

peripherally after *in vivo* 24-hour icv propranolol pre-treatments (see Section 3-3-E, and Figures 32 and 33), the pre-treatments had no impact on hepatic CYP2D activity. The increase in severity of hypothermia in both TG and WT after inhibiting CYP2D in the brain (*in vivo* 4-hour pre-treatment with icv propranolol) suggests that CYP2D in the brain protects against harmine-induced neurotoxicity. Moreover, the increase in severity of hypothermia in TG after the irreversible and selective inhibition of human CYP2D6 in the brain (*in vivo* 24-hour pre-treatment with icv propranolol) suggests that human CYP2D6 in the brain has sufficient activity *in vivo* to protect against harmine-induced neurotoxicity. These additional experiments provide 1) evidence that this model that we have developed is not limited to investigating the role that human CYP2D6 in the brain plays in drug response and drug levels of clinical drugs such as haloperidol but can also be used to investigate the role that human CYP2D6 in the brain plays in protecting against neurotoxins such as harmine.

4-6 Conclusions

In conclusion, we found a) acute haloperidol-induced catalepsy is decreased after inhibiting CYP2D in brain, and increased after inducing CYP2D in brain, b) chronic haloperidol-induced vacuous chewing movements are increased after inhibiting CYP2D in brain. We developed and applied an animal model using a CYP2D6 humanized TG mouse (vs WT) and demonstrated c) TG (versus WT) have faster *ex vivo* CYP2D-mediated dextrorphan formation rate in the liver and in the brain, d) *in vitro* propranolol decreases *ex vivo* CYP2D-mediated dextrorphan formation rate in TG, WT, and human liver microsomes, e) *in vivo* 24-hour pre-treatment with ip propranolol decreases *in vivo* plasma dextrorphan/dextromethorphan ratio and *ex vivo* dextrorphan formation rate by liver microsomes, and increases *in vivo* plasma haloperidol levels and acute haloperidol-induced catalepsy in TG and WT, f) in TG, *in vivo* 24-hour pre-treatment with icv propranolol decreases *in vivo* brain dextrorphan/dextromethorphan ratio and *ex vivo* dextrorphan formation rate by brain membranes, and decreases acute haloperidol-induced catalepsy, g) in WT, *in vivo* 4-hour pre-treatment with icv propranolol decreases *in vivo* brain dextrorphan/dextromethorphan ratio but not *ex vivo* dextrorphan formation rate by brain membranes. Neither *in vivo* 4- or 24-hour icv propranolol pre-treatment changed *in vivo* plasma dextrorphan/dextromethorphan ratio and *ex vivo* dextrorphan formation rate by liver microsomes. Finally, we applied this *in vivo* 24-hour icv propranolol pre-treatment model to harmine-induced neurotoxicity, demonstrating a role for CYP2D, and specifically human CYP2D6. Together these acute and chronic treatment studies demonstrated a role for CYP2D, and human CYP2D6, in the brain of living animals in both adverse drug responses and neurotoxicity.

Specific manipulation of CYP2D in rat brain or human CYP2D6 in TG brain is useful for understanding the role that CYP2D in the brain plays in drug-induced therapeutic effects and

adverse events. In rats, we provided evidence to indicate that acute haloperidol-induced catalepsy is caused by a neurotoxic haloperidol metabolite formed in the brain by CYP2D. Further, we demonstrated that CYP2D in the brain alters chronic haloperidol-induced vacuous chewing movements, likely related to altering brain levels of the parent compound haloperidol. In mice, the model that we have developed allows for both the *in vivo* study of CYP2D in the brain as well as the focused study of specifically human CYP2D6 in the brain. Using this model, we have shown that human CYP2D6 in the TG brain is sufficient to change haloperidol concentrations and acute haloperidol-induced catalepsy. This is the first demonstration that human CYP2D6 in brain contributes meaningfully to drug metabolism and response *in vivo*. This work was extended to harmine-induced neurotoxicity. In conclusion, variation in human CYP2D6 activity in the brain, whether due to genetics, age, or exposure to exogenous compounds, may result in changes to localized metabolism of clinically used drugs and neurotoxins.

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Copyright Acknowledgements

Appendices

Appendix A: Human CYP2D6 in the brain is protective against harmine-induced neurotoxicity: evidence from humanized CYP2D6 transgenic mice

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*These authors contributed equally: Marlaina R Stocco and Cole Tolledo

When this thesis was submitted, this paper was not yet published. Cole Tolledo, along with other authors, designed the study. Cole Tolledo optimized the *ex vivo* brain and liver incubation experiments, and performed the *ex vivo* liver incubation experiments. Cole Tolledo and Marlaina Stocco performed the *ex vivo* brain incubation experiments. Cole Tolledo, Marlaina Stocco, and Fariba Baghai Wadji performed the cannulation surgeries. Cole Tolledo analyzed all of the *ex vivo* brain and liver incubation data. All authors critically reviewed, edited, and approved the final version of the publication. Frank Gonzalez provided the CYP2D6-transgenic mice.

Abstract

Background CYP2D6 metabolically inactivates several neurotoxins, including beta-carbolines, which are implicated in neurodegenerative diseases. Variation in CYP2D6 within the brain may alter local inactivation of neurotoxic beta-carbolines, thereby influencing neurotoxicity. The beta-carboline harmine, which induces hypothermia and tremor, is metabolized by CYP2D6 to the non-hypothermic/non-tremorgenic harmol. Transgenic mice (TG), expressing human CYP2D6 in addition to their endogenous mouse CYP2D, experience less harmine-induced hypothermia and tremor compared to wild type mice (WT).

Methods In brain, propranolol is an irreversible inhibitor of human CYP2D6 and a competitive inhibitor of mouse CYP2D. To elucidate the role of CYP2D in general within the brain, a 4-hour intracerebroventricular (ICV) pretreatment with propranolol (or vehicle) was given to TG and WT, and harmine-induced hypothermia and tremor responses were assessed. To specifically demonstrate that human CYP2D6 expressed in TG brain altered harmine response severity, a 24-hour ICV propranolol (or vehicle) pretreatment was given to TG and WT, and harmine-induced hypothermia and tremor responses were assessed. Pretreatment effects were analyzed using Repeated Measures ANOVAs to compare response-time curves and Bonferroni adjusted t-tests to compare area under the curve and mean response.

Results The 4-hour ICV propranolol pretreatment increased harmine-induced hypothermia and tremor in TG and increased harmine-induced hypothermia in WT. The 24-hour ICV propranolol pretreatment increased harmine-induced hypothermia in TG with no effect in WT, which confirmed that there were no off-target effects of ICV propranolol pretreatment. As previously shown, neither the 4-hour nor 24-hour ICV propranolol pretreatments had an effect on liver CYP2D activity in TG or in WT.

Conclusions CYP2D activity in general within the brain was sufficient *in vivo* to alter harmine response. Further, human CYP2D6 activity specifically in TG brain was sufficient *in vivo* to mitigate harmine-induced neurotoxicity. These findings suggest that human CYP2D6 in the brain is protective against beta-carboline-induced neurotoxicity and that the extensive interindividual variability in CYP2D6 expression in human brain may contribute to variation in susceptibility to certain neurotoxin-associated neurodegenerative disorders.

Keywords

CYP2D6; drug metabolism; neurotoxicity; harmine; propranolol

List of abbreviations

CYP, Cytochrome P450

MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

TG, transgenic mice

WT, wild type mice

ICV, intracerebroventricular

IP, intraperitoneal

RM, repeated measures

AUC, area under the curve

NAA/tCR, N-acetylaspartate/total creatine

1. Background

Members of the cytochrome P450 enzyme (CYP) family catalyze the oxidative metabolism of most drugs and toxins (1). CYP2D is a CYP subfamily, which includes CYP2D6 in humans and various CYP2D isoforms in other species (2). CYP2D metabolizes approximately 25% of all clinically used drugs, many of which are centrally acting, including opioids, psychostimulants, and antipsychotics (3, 4). Additionally, CYP2D metabolizes neurotoxins, including tetrahydroisoquinolines (5), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (6), and beta-carbolines (7), which are causally implicated in neurodegeneration (8-10). Human *CYP2D6* is highly genetically polymorphic; variation in *CYP2D6* is associated with differences in drug pharmacokinetics and resultant drug response (3, 4), as well as with differences in susceptibility to neurodegenerative diseases (11, 12). While CYP2D is expressed at high levels in the liver, CYP2D is also expressed and enzymatically active in the brain (13, 14), where local metabolism can alter brain levels of substrates (e.g. centrally acting drugs and neurotoxins) and their metabolites (15, 16). Hepatic CYP2D is considered uninducible, but CYP2D in the brain is readily induced by exposure to xenobiotics including chronic nicotine (17, 18). CYP2D6 variation in the human brain, in addition to variation in the liver, may influence drug-induced neurotoxicity and neurodegeneration by changing levels of neurotoxins and their metabolites within the brain.

Exposure to beta-carbolines can occur through consumption of certain foods, alcohol, and inhalation of tobacco smoke (19, 20). Elevated levels of certain beta-carbolines are positively correlated with measures of neurodegeneration (21) and are associated with neurodegenerative diseases including Parkinson’s disease (22) and Essential tremor (23). Harmine is a beta-carboline that induces hypothermia (24) and tremor (25), which serve as

measures of neurotoxicity in rodent models of neurodegenerative disorders (26). CYP2D6 metabolism constitutes a major inactivation pathway for many beta-carbolines including harmine, which is metabolized by CYP2D6 to the inactive metabolite harmol (7, 27). This suggests that CYP2D6 may protect against beta-carboline-induced neurotoxicity. Humanized CYP2D6-expressing transgenic mice (TG), which express human CYP2D6 in addition to mouse CYP2D, metabolize harmine (and related compounds) more rapidly than wild type mice (WT) (7); they also exhibit less severe hypothermia (28) and tremor (29) responses, consistent with a protective role for CYP2D6 metabolism. Harmine readily enters and distributes throughout the brain (30), suggesting a potential important contribution of CYP2D6- and CYP2D-mediated metabolism in the brain to mitigating neurotoxicity.

In cultured human neurons, inhibiting CYP2D6 increased MPTP-induced neurotoxicity *in vitro* (31, 32). CYP2D6 protein was lower in human brains of those with Parkinson's disease compared to brains from age-matched healthy controls, even after controlling for *CYP2D6* genotype (33). This is consistent with lower CYP2D6 in the human brain reducing protection against xenobiotic-induced neurotoxicity and neurodegeneration, while elevated CYP2D6 in brain may be protective. We hypothesized a role for CYP2D6 within the brain, whereby local CYP2D6-mediated beta-carboline inactivation may mitigate beta-carboline-induced neurotoxicity. The aims of the current study were 1) to assess the role of CYP2D in general within the brain in neurotoxicity-related responses following administration of the beta-carboline harmine, and 2) to demonstrate specifically that human CYP2D6 expressed within the brain was sufficient to mitigate harmine-induced neurotoxicity. Selective pharmacological manipulation of CYP2D in the brain provides an effective tool to study the impact of CYP2D metabolism in the brain (15, 16). We recently developed novel approaches, using these TG and WT, to selectively inhibit human CYP2D6

and/or mouse CYP2D in the brain, without impacting hepatic metabolism in either mouse line (34). Propranolol acts as an irreversible inhibitor of human CYP2D6 expressed in TG brain and a competitive inhibitor of mouse CYP2D in TG and WT brain (34). To assess the role of CYP2D in general within the brain in harmine-induced hypothermia and tremor responses (Aim 1), we used a 4-hour intracerebroventricular (ICV) propranolol pretreatment, which irreversibly inhibits human CYP2D6 in the TG brain and competitively inhibits mouse CYP2D in the brain of TG and WT (Fig. 1) (34). To study the impact of human CYP2D6 specifically in the brain (Aim 2), we used a 24-hour ICV propranolol pretreatment, which selectively and irreversibly inhibits human CYP2D6 in TG brain but does not inhibit mouse CYP2D in the brain of TG or WT (i.e. there is no competitive inhibition remaining 24 hours after propranolol pretreatment) (Fig. 1) (34). This novel approach was used to demonstrate both the general role for CYP2D in the brain and, for the first time, that human CYP2D6 expressed in the brain of a mammal is sufficient to alter response to a neurotoxin, specifically the beta-carboline harmine.

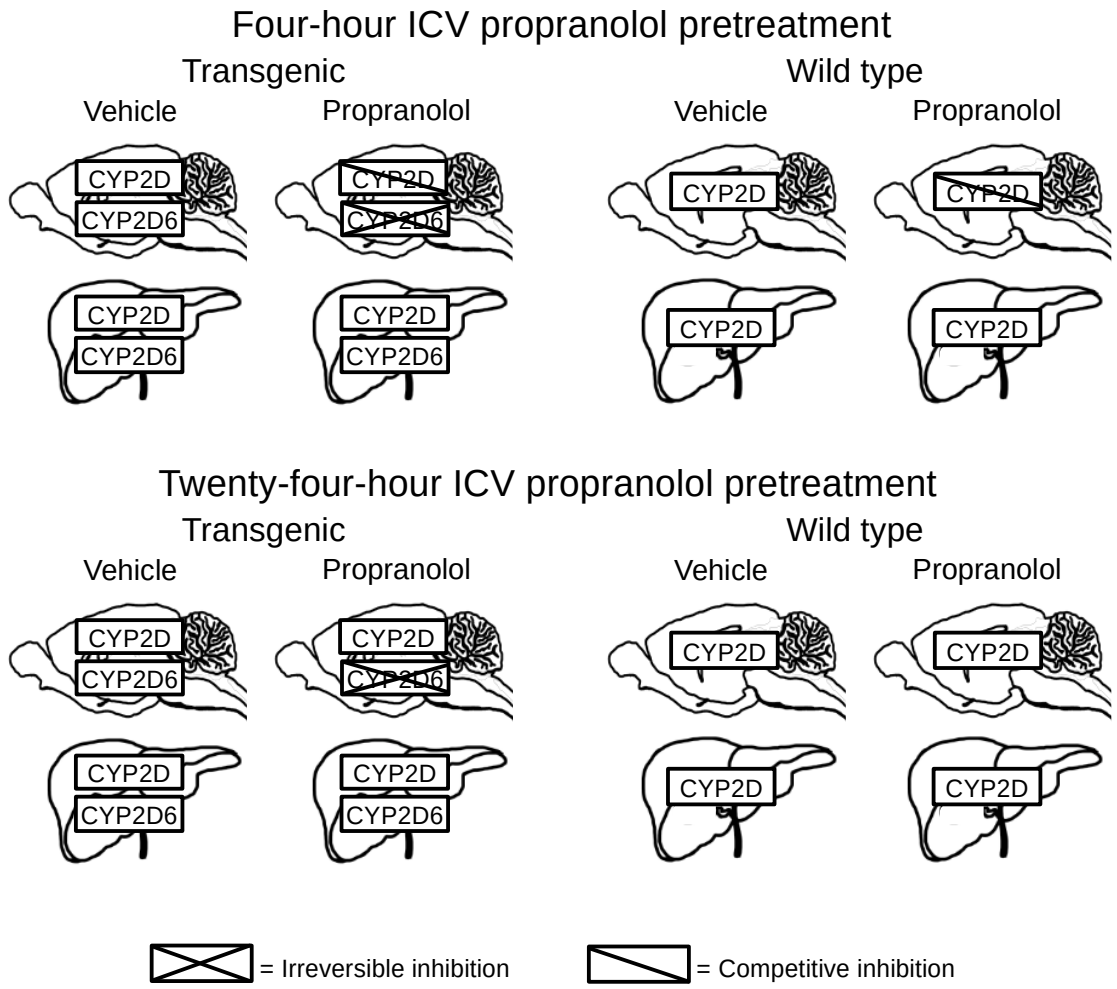

Figure 1. Pretreatment effects on CYP2D and CYP2D6 in brain and liver of TG and WT. Four-hour ICV propranolol pretreatment irreversibly inhibits human CYP2D6 in TG brain and competitively inhibits mouse CYP2D in TG and WT brain. Twenty-four-hour ICV propranolol pretreatment irreversibly inhibits human CYP2D6 in TG brain but no longer inhibits mouse CYP2D in TG or WT brain. Following four-and twenty-four-hour ICV propranolol pretreatments, there is no inhibition of human CYP2D6 in TG liver, or of mouse CYP2D in TG or WT liver, indicating propranolol given ICV does not reach the liver in sufficient amounts to alter hepatic metabolism.

2. Methods

2.1. Animals

TG were produced by microinjecting fertilized FVB/N mouse eggs with an insert that included the human *CYP2D6* gene sequence (exon 1-9), the 5'- and 3'- flanking sequences,

and the pseudogenes *CYP2D7P1 and CYP2D8P1* (Genbank accession number BX247885, PAC clone RP4-669P10) (29). TG founders underwent successive matings with C57BL/6J mice, and polymerase chain reaction and Southern blot analyses were used to confirm the incorporation of the full length *CYP2D6* gene (29). For these experiments, all TG were homozygous and this was confirmed by genotyping prior to use, as previously described (35). Age-matched adult male TG (29) and WT (Charles River, Saint-Constant, QC, Canada) mice were housed in groups of 1-4, given food and water ad libitum, and kept under a 12-hour light/dark cycle with testing during the light phase. All procedures were approved by the Animal Care Committee at the University of Toronto and were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the National Institutes of Health.

2.2. Intracerebroventricular (ICV) cannulation surgery

Mice were anesthetized with 2% isofluorane and implanted with 26 gauge stainless steel guide cannulas into the right lateral ventricle (anterior-posterior -1.0 mm, lateral -0.5 mm, from bregma, and dorsoventral -2.2 mm) (36). Guide cannulas were secured using dental cement and small stabilizing screws. Dummy cannulas were inserted after surgery. Animals recovered for at least 7 days prior to experimentation.

2.3. Drugs and drug administration

As previously described (34), propranolol hydrochloride (Sigma, Oakville, ON, Canada) was dissolved in its vehicle, a 20% (w/v) solution of 2-hydroxypropyl-β-cyclodextrin (Sigma, Oakville, ON, Canada) in distilled water, to a final concentration of 40 µg propranolol base/µl cyclodextrin vehicle solution. A total volume of 2 µl (80 µg propranolol total or cyclodextrin vehicle) was injected ICV at a rate of 1 µl/minute through

the guide cannula with an automated injector system (Harvard Apparatus Pump 11 Pico Plus Elite, Holliston, MA, USA). Following the ICV injection, mice rested for 1 minute before injectors were removed and cannula dummies replaced. ICV pretreatment injections were given either 24 or 4 hours prior to harmine treatment. Harmine hydrochloride (Sigma, Oakville, ON, Canada) was dissolved in distilled water. Harmine (or distilled water vehicle) was injected intraperitoneally (IP) at a volume of 0.1 ml/kg body weight. Harmine doses of 5.0, 7.5, and 10.0 mg base/kg were tested in the dose response experiments (n = 6-8 per dose, per mouse line) and 7.5 mg/kg harmine base was used in subsequent experiments with ICV pretreatments.

2.4. Harmine-induced hypothermia and tremor assessment

Hypothermia was calculated as the change in body temperature compared to a baseline, where baseline was measured 15 minutes prior to harmine injection. Body temperature was measured using a digital thermometer and lubricated thermistor probe inserted 1.0 cm rectally. Tremor was scored based on 30 seconds of constant observation per time point and using a modified version of a previously published scale (29). Briefly, this included whole number scores of 0 (no tremor), 1.0 (mild infrequent tremor), 2.0 (modest intermittent tremor), 3.0 (severe frequent tremor), and 4.0 (severe constant tremor) and scoring was performed using half unit increments (e.g. 0.5, 1.0, 1.5).

2.5. Four-hour ICV pretreatment and harmine-induced hypothermia and tremor

A pilot study was conducted in WT (n = 12) and the pretreatment effect size for hypothermia was used to derive the WT sample size (n = 8) needed; TG sample size (n = 16) was obtained by doubling that of the WT. TG and WT received ICV propranolol (or

cyclodextrin vehicle) pretreatment 4 hours prior to harmine (or distilled water vehicle) IP treatment. To obtain within-animal data, ICV pretreatment and IP treatment conditions were crossed over (i.e. each mouse was tested in each of the four combinations: propranolol/harmine, vehicle/harmine, propranolol/vehicle, and vehicle/vehicle). Each test session was separated by a 7-day washout; the order was randomized and counterbalanced. Hypothermia and tremor were assessed at baseline and at 15-minute intervals for 90 minutes.

2.6. Twenty-four-hour ICV pretreatment and harmine-induced hypothermia and tremor

Sample sizes were increased in this experiment to account for a smaller predicted effect size in TG, compared to the effect size observed following 4-hour pretreatment. TG (n = 25) and WT (n = 24) mice received ICV propranolol (or cyclodextrin vehicle) pretreatment 24 hours prior to IP harmine treatment. To obtain within-animal data, ICV pretreatment conditions were crossed over after a 7-day washout; the order was randomized and counterbalanced. Hypothermia and tremor were assessed at baseline and at 15-minute intervals for 90 minutes.

2.7. Four- and twenty-four-hour ICV pretreatment and in vitro brain and liver CYP2D activity

Four- or twenty-four-hour ICV propranolol (or cyclodextrin vehicle) pretreatment was administered to TG and WT (n = 5-6 per pretreatment per mouse line for four- and twenty-four-hour experiments). Mice were then euthanized, and cerebellums and livers were collected. Brain (i.e. cerebellum) membrane preparation and incubations were performed on the same day, while livers were stored at -80˚C with microsome preparation and incubations performed on a subsequent day (35).

Total membranes were prepared from cerebellum and microsomal membranes were prepared from liver as previously described (35, 37, 38). Dextromethorphan hydrobromide (Sigma, Oakville ON, Canada) was used as a CYP2D probe substrate in both brain and liver incubations; dextromethorphan undergoes CYP2D-specific O-demethylation to dextrorphan (39). Incubation conditions were optimized for linear dextrorphan formation by mouse brain membranes and liver microsomes (34, 35). For brain, membranes prepared fresh from whole cerebellums (300-400 µg) were incubated with 50 µM dextromethorphan (approximate V_{max}) and 1 mM NADPH in artificial cerebrospinal fluid (pH 7.4) for 90 minutes and at 37˚C under 95% O_2/5% CO_2 in a final volume of 1 ml (34). For liver, microsomes (50 µg protein) were incubated with 5 µM dextromethorphan (approximate K_m) and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) for 10 minutes and at 37˚C in a final volume of 0.5 ml (35). Incubation reactions were started by adding dextromethorphan, and reactions were stopped by adding an equal volume of a hexane-butanol (95:5 v/v) solution. Immediately before extraction, 5 ng dextrorphan base (dextrorphan tartrate, Sigma, Oakville ON, Canada) was added to each brain incubate; this was added to ensure that dextrorphan concentrations were above the limit of quantification (34). This was subsequently subtracted from the concentration measured in each sample to calculate enzymatically formed dextrorphan. All samples were then extracted, the organic layer collected and dried under nitrogen, and the residue dissolved in mobile phase for analysis by high performance liquid chromatography, as previously described (16, 34, 35).

2.8. Statistical analysis

All analyses were performed using Prism6 (GraphPad version 6.0c, La Jolla, California, USA) software, and all outliers were included in original analyses. Mixed-design

ANOVAs were used to compare responses (i.e. either hypothermia or tremor) across time, for each IP treatment (i.e. mouse line x pretreatment, within harmine or distilled water IP treatment); subsequent analyses were only performed when there was a significant effect of group (i.e. mouse line x pretreatment). Repeated-measures (RM) ANOVAs were then used to compare responses between ICV propranolol and vehicle pretreatments within mouse line, and mixed-design ANOVAs were used to compare responses between vehicle-pretreated TG and WT. Area under the response-time curve ($AUC_{0\text{-}90}$) was analyzed across all groups (mouse line x pretreatment) using two-factor mixed-design ANOVAs. $AUC_{0\text{-}90}$ was compared between ICV propranolol and vehicle pretreatments (within mouse line) using Bonferroni-adjusted paired two-tailed t-tests, and between vehicle-pretreated TG and WT using unpaired two-tailed t-tests. Mean response was calculated by dividing $AUC_{0\text{-}90}$ by the recorded response duration (90 minutes). Mean response was compared between ICV propranolol and vehicle pretreatments (within mouse line) using Bonferroni-adjusted paired two-tailed t-tests. Dextrorphan formation (i.e. CYP2D enzymatic activity for brain and for liver) was compared between ICV propranolol and vehicle pretreatments (within mouse line) using unpaired two-tailed t-tests. For all unpaired t-tests, Welch's correction was applied when the F-test comparing group variance was significant. Between-animal data is graphed showing standard deviation and within-animal data is graphed showing standard error of the mean.

3. Results

3.1. Harmine hypothermia and tremor dose response

Harmine dose-dependently induced hypothermia in TG (dose, $F(3,23) = 23.0$, $p < 0.0001$) (Fig. 2A) and in WT (dose, $F(3,20) = 48.0$, $p < 0.0001$) (Fig. 2B). Harmine dose-

dependently induced tremor in TG (dose, $F(2,20) = 7.61$, $p = 0.0035$) (Fig. 2C) and in WT (dose, $F(2,18) = 15.7$, $p = 0.0001$) (Fig. 2D). The 7.5 mg/kg harmine dose was selected for subsequent studies, because it allowed for the detection of an increase or decrease in response in TG and WT.

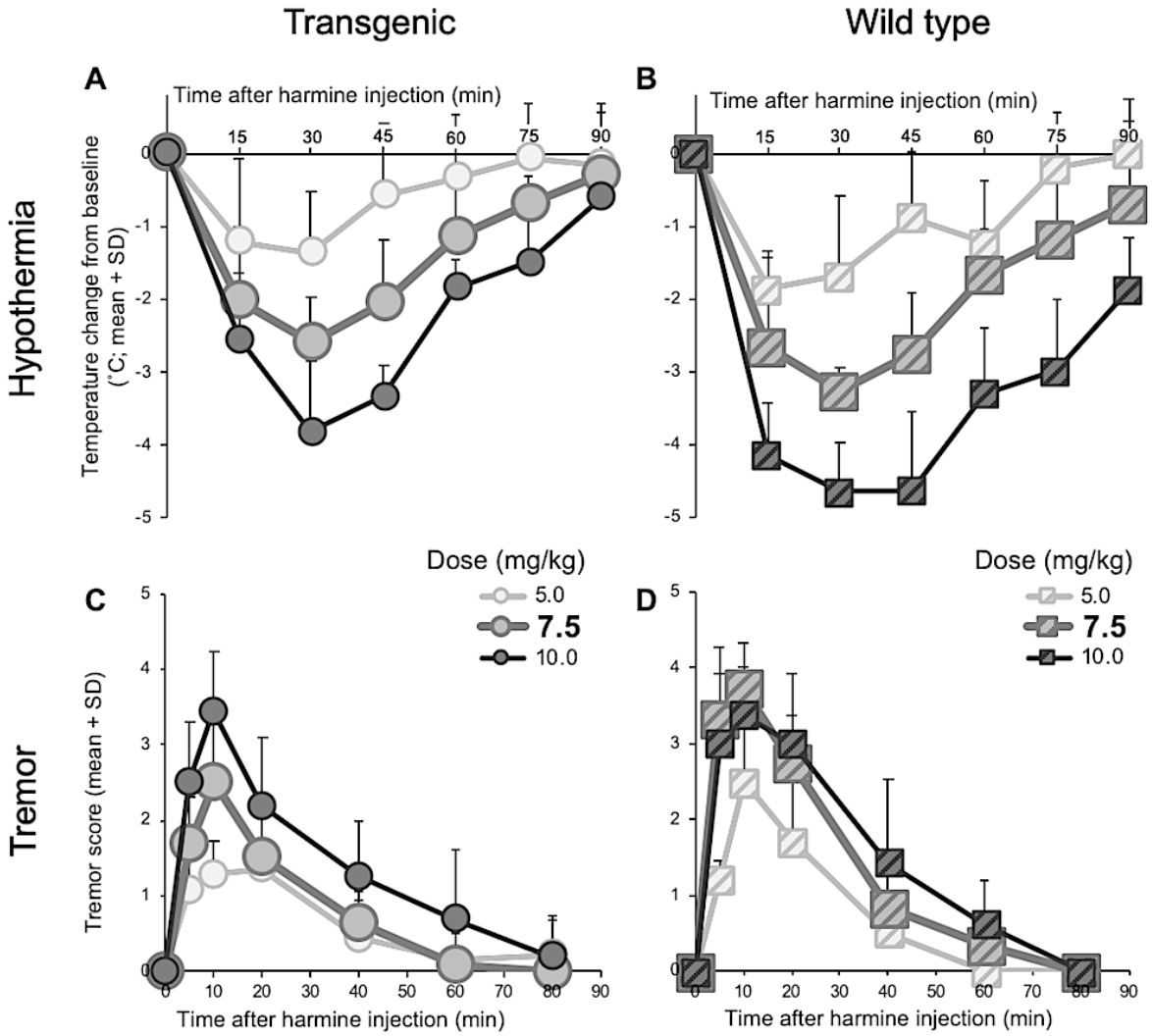

Figure 2. Harmine dose-dependently induced hypothermia and tremor in TG and WT. Change in body temperature from baseline after injection with harmine (5.0, 7.5, or 10.0 mg/kg IP) of (**A**) TG and (**B**) WT. Tremor score after injection with harmine (5.0, 7.5, or 10.0 mg/kg IP) in (**D**) TG and (**E**) WT. Symbols for the harmine 7.5 mg/kg dose effects are enlarged, to illustrate the effects of the dose that was used in subsequent experiments. SD, standard deviation.

3.2. Four-hour ICV propranolol pretreatment exacerbated harmine-induced hypothermia in TG and in WT.

Four-hour ICV propranolol (versus vehicle) pretreatment increased harmine-induced hypothermia in TG (pretreatment, $F(1,15) = 94.4$, $p < 0.0001$) and in WT (pretreatment, $F(1,7) = 13.4$, $p = 0.0081$) (Fig. 3A). There was a main effect of pretreatment on hypothermia, evaluated by $AUC_{0\text{-}90}$ ($F(1,22) = 75.0$, $p < 0.0001$), due to propranolol (versus vehicle) pretreatment increasing $AUC_{0\text{-}90}$ in TG ($t(15) = 9.42$, $p < 0.0001$) and in WT ($t(7) = 3.91$, $p = 0.0116$) (Fig. 3B). In propranolol (versus vehicle) pretreated TG, the increase in mean response ($t(15) = 9.57$, $p < 0.0001$) corresponded to a 1.7-fold increase in apparent harmine dose (Fig. 3C). In propranolol (versus vehicle) pretreated WT, the increase in mean response ($t(7) = 3.79$, $p = 0.0136$) corresponded to a 1.3-fold increase in apparent harmine dose (Fig. 3D).

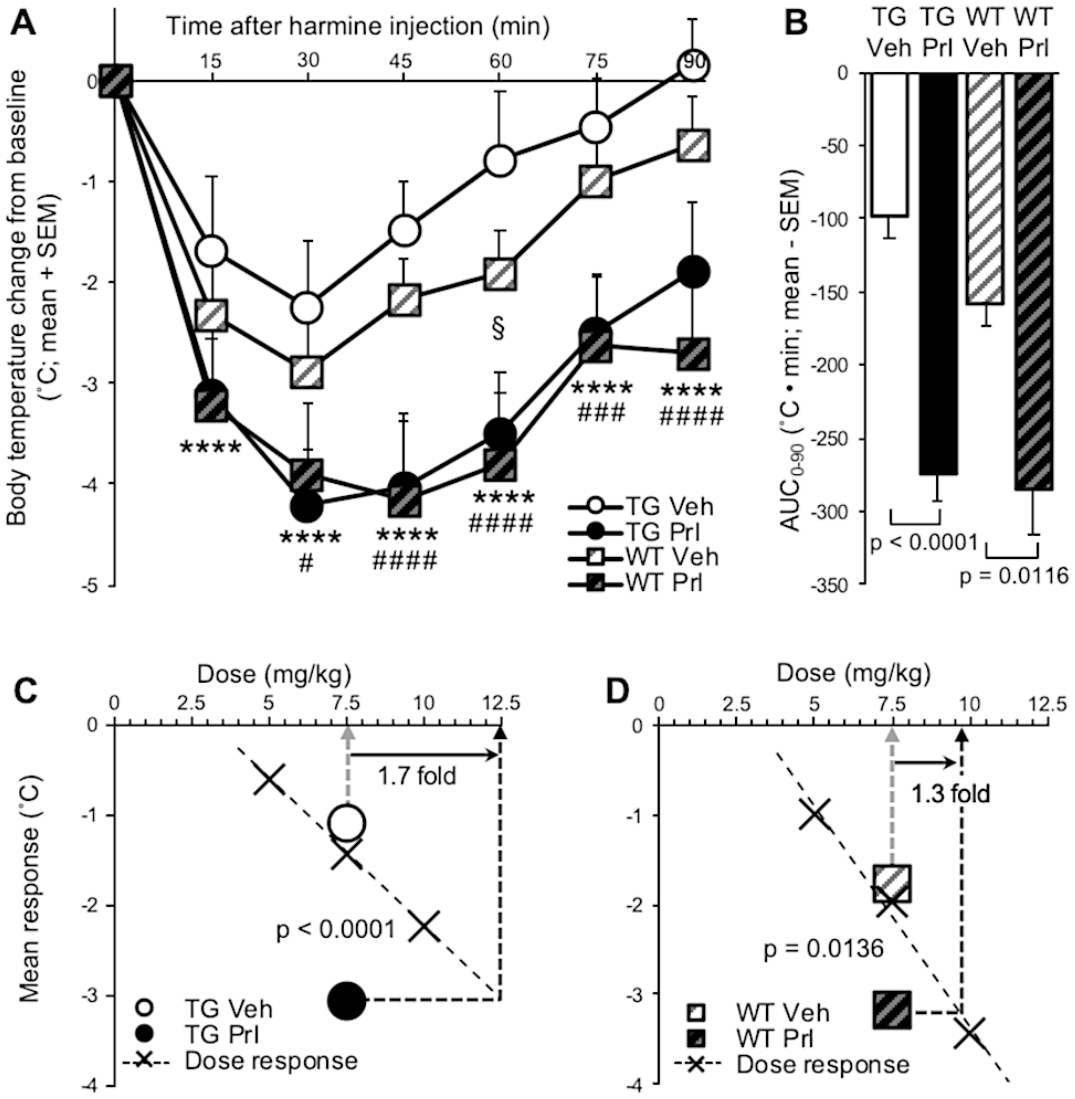

Figure 3. Four-hour ICV propranolol (versus vehicle) pretreatment exacerbated harmine-induced hypothermia in TG and WT. A) Four hours after ICV propranolol or vehicle pretreatment, harmine treatment was administered and change in body temperature from baseline was recorded for 90 minutes in TG (n=16) and WT (n=8). **B)** $AUC_{0\text{-}90}$ of ICV propranolol or vehicle pretreated TG and WT. Mean response of ICV propranolol or vehicle pretreated (**C**) TG and (**D**) WT; values are superimposed on dose response data from Fig 3A and 3B respectively. Propranolol versus vehicle pretreated TG: ***p < 0.001, ****p < 0.0001; propranolol versus vehicle pretreated WT: #p < 0.05, ###p < 0.001, ####p < 0.0001; vehicle pretreated WT versus TG: §p < 0.05. Veh, vehicle; Prl, propranolol; SEM, standard error of the mean; AUC, area under the curve.

Hypothermia was more severe in vehicle-pretreated WT compared to TG (mouse line, $F(1,22) = 6.69$, $p = 0.0168$) (Fig. 3A). IP distilled water treatment had no effect on body

temperature, and body temperature following IP distilled water did not differ between ICV pretreatment groups or mouse lines (ANOVA, all $p > 0.05$) (Fig 4).

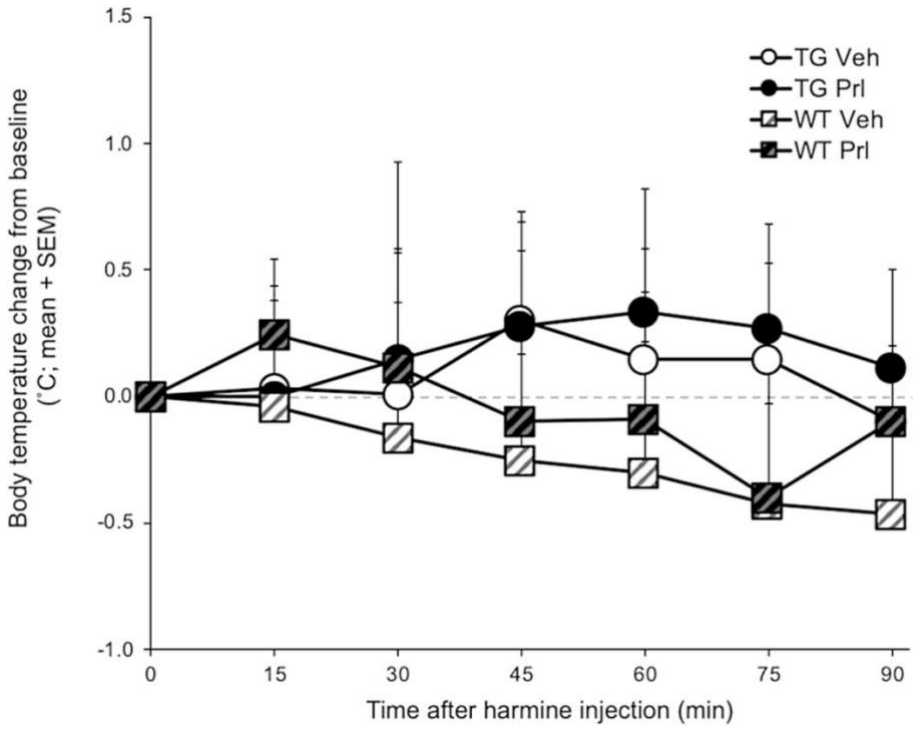

Figure 4. Four-hour ICV propranolol (versus vehicle) pretreatment had no effect on body temperature. Four hours after ICV propranolol or vehicle pretreatment, IP distilled water treatment was administered and change in body temperature from baseline was recorded for 90 minutes in TG (n=16) and WT (n=8). There was no effect of pretreatment in TG or WT, and there was no effect of mouse line (RM and mixed-design ANOVAs, all $p > 0.05$). Veh, vehicle; Prl, propranolol; SEM, standard error of the mean.

3.3. Twenty-four-hour ICV propranolol pretreatment exacerbated harmine-induced hypothermia in TG, but not in WT.

Twenty-four-hour ICV propranolol (versus vehicle) pretreatment increased harmine-induced hypothermia in TG (pretreatment, $F(1,24) = 49.7$, $p < 0.0001$), but had no effect in WT (Fig. 5A). There was an interaction effect of pretreatment and mouse line on hypothermia, evaluated by $AUC_{0\text{-}90}$ ($F(1,47) = 12.2$, $p = 0.001$), as well as a main effect of

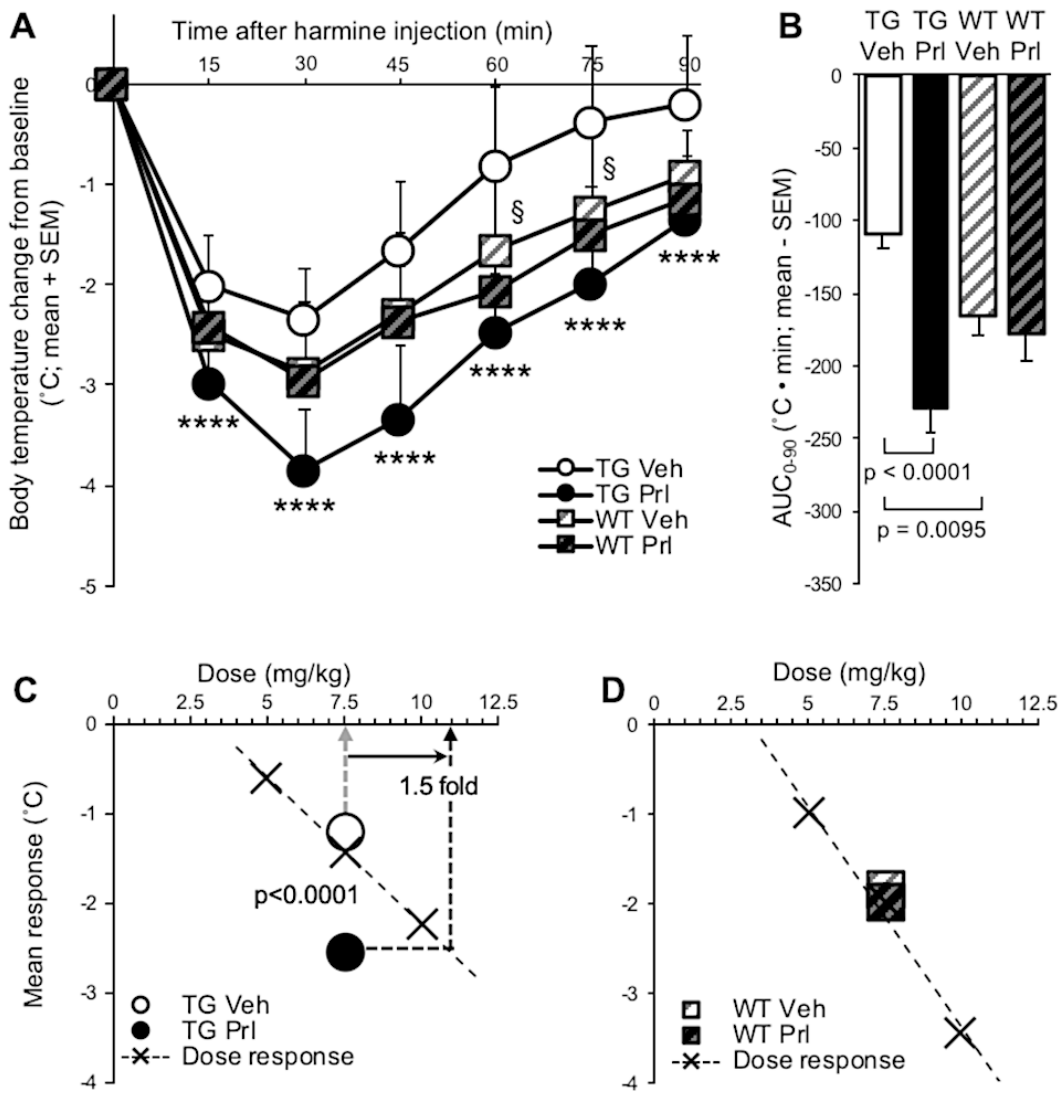

Figure 5. Twenty-four-hour ICV propranolol (versus vehicle) pretreatment exacerbated harmine-induced hypothermia in TG. A) Twenty-four hours after ICV propranolol or vehicle pretreatment, harmine treatment was administered and change in body temperature from baseline was recorded for 90 minutes in TG (n=25) and WT (n=24). **B)** $AUC_{0\text{-}90}$ of ICV propranolol or vehicle pretreated TG and WT. Mean response of ICV propranolol or vehicle pretreated (**C**) TG and (**D**) WT; values are superimposed on dose response data from Fig 3A and 3B respectively. Propranolol versus vehicle pretreated TG: ***$p < 0.001$, ****$p < 0.0001$; vehicle pretreated WT versus TG: §$p < 0.05$. Veh, vehicle; Prl, propranolol; SEM, standard error of the mean; AUC, area under the curve.

pretreatment ($F(1,47) = 19.1$, $p < 0.0001$), due to propranolol (versus vehicle) increasing $AUC_{0\text{-}90}$ in TG ($t(24) = 7.06$, $p < 0.0001$) (Fig. 5B). In propranolol (versus vehicle) pretreated TG, the increase in mean response ($t(24) = 7.06$, $p < 0.0001$) corresponded to a 1.5-fold

increase in apparent dose (Fig. 5C). In propranolol (versus vehicle) pretreated WT, there was no change in mean response (Fig. 5D).

Hypothermia was more severe in vehicle-pretreated WT compared to TG (mouse line, $F(1,47) = 8.09$, $p = 0.0066$) (Fig. 5A). The $AUC_{0\text{-}90}$ was greater in vehicle-pretreated WT compared to TG ($t(38.47) = 2.73$, $p = 0.0095$) (Fig. 5B).

3.4. Four-hour ICV propranolol pretreatment exacerbated harmine-induced tremor in TG, but not in WT.

Four-hour ICV propranolol (versus vehicle) pretreatment increased harmine-induced tremor in TG (pretreatment, $F(1,15) = 24.3$, $p = 0.0002$), but not in WT (Fig. 6A). There was a main effect of pretreatment on tremor, evaluated by $AUC_{0\text{-}90}$ ($F(1,22) = 9.61$; $p = 0.0052$), due to propranolol (versus vehicle) pretreatment increasing $AUC_{0\text{-}90}$ in TG ($t(15) = 4.96$, $p = 0.0004$) (Fig. 6B). In propranolol (versus vehicle) pretreated TG, the increase in mean response ($t(15) = 4.82$, $p = 0.0004$) corresponded to a 1.4-fold increase in apparent dose (Fig. 6C). In propranolol (versus vehicle) pretreated WT, there was no change in mean response (Fig. 6D). Propranolol (versus vehicle) pretreatment also increased peak tremor (measured at 15 minutes after harmine injection) in TG ($t(15) = 4.20$, $p = 0.0008$) (Fig 7A), but not in WT (Fig 7B).

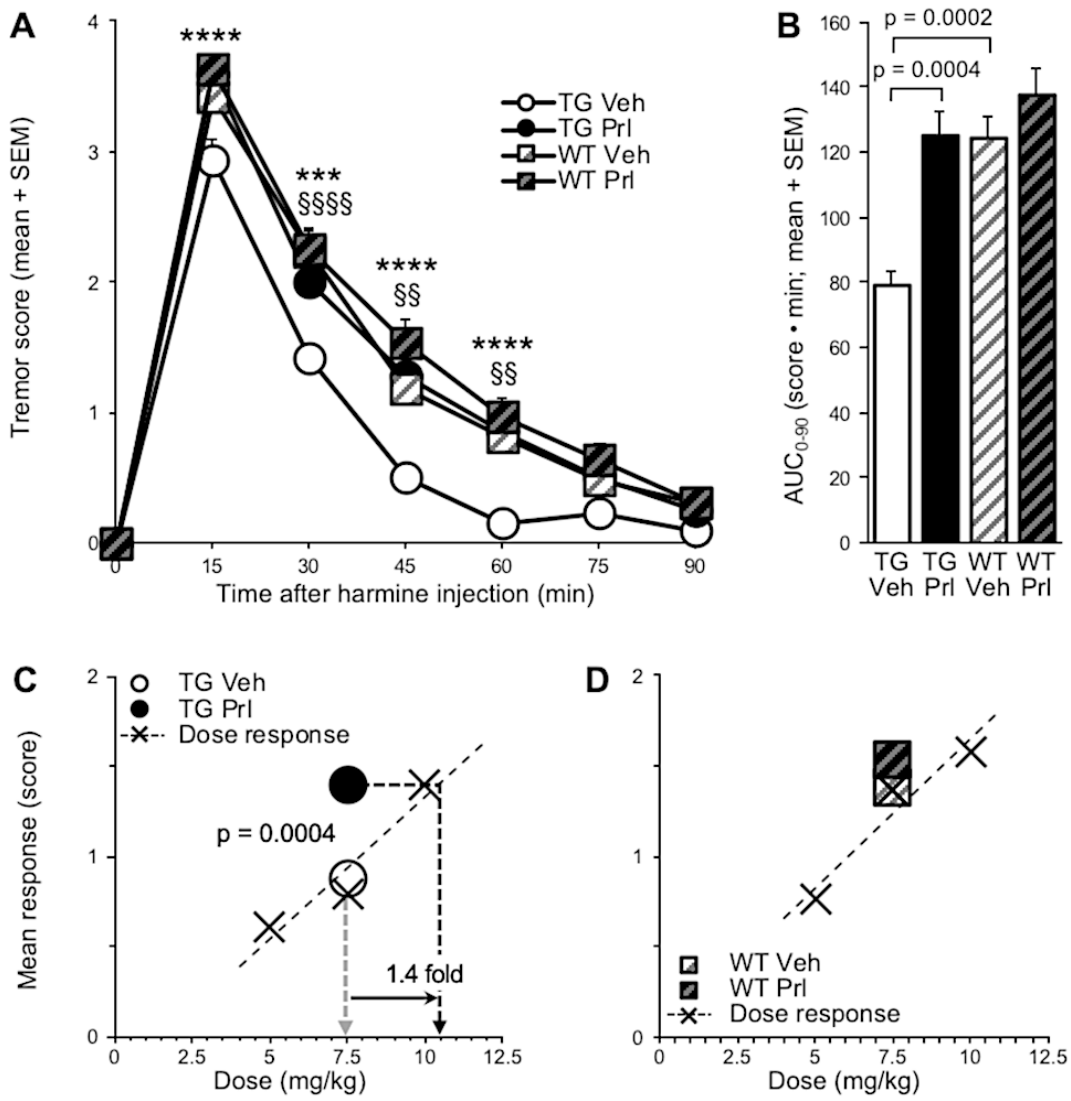

Figure 6. Four-hour ICV propranolol (versus vehicle) pretreatment exacerbated harmine-induced tremor in TG. A) Four hours after ICV propranolol or vehicle pretreatment, harmine or distilled water (data not shown) treatment was administered, and tremor was scored for 90 minutes in TG (n=16) and WT (n=8). **B)** $AUC_{0\text{-}90}$ of ICV propranolol or vehicle pretreated TG and WT. Mean response of ICV propranolol or vehicle pretreated (**C**) TG and (**D**) WT; values are superimposed on dose response data from Fig 3C and 3D respectively. Propranolol versus vehicle pretreated TG: ***p < 0.001, ****p < 0.0001; vehicle pretreated WT versus TG: §§p < 0.01, §§§§p < 0.0001. Veh, vehicle; Prl, propranolol; SEM, standard error of the mean; AUC, area under the curve.

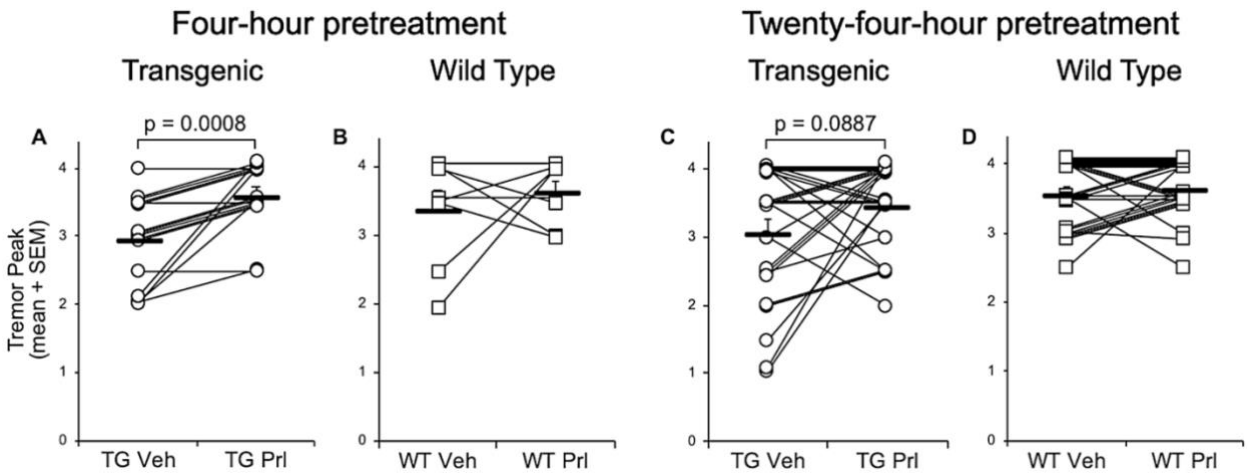

Figure 7. ICV propranolol (versus vehicle) pretreatment increased peak tremor in TG. Peak tremor (score at 15 minutes after harmine injection) after four-hour pretreatment in (**A**) TG (n=16) and (**B**) WT (n=8). Propranolol (versus vehicle) pretreatment increased peak tremor in TG ($t(15) = 4.20$, $p = 00008$), but not in WT. Peak tremor after twenty-four-hour pretreatment in (**C**) TG (n=25) and (**D**) WT (n=24). Propranolol (versus vehicle) trended towards increasing peak tremor in TG ($t(24) = 1.77$, $p = 0.0887$), but had no effect in WT. Veh, vehicle; Prl, propranolol; SEM, standard error of the mean

Tremor was more severe in vehicle-pretreated WT compared to TG (mouse line, $F(1,22) = 20.4$, $p = 0.0002$) (Fig. 6A). There was a main effect of mouse line on tremor, evaluated by $AUC_{0\text{-}90}$ ($F(1,22) = 13.6$, $p = 0.0013$), due to $AUC_{0\text{-}90}$ being greater in vehicle-pretreated WT compared to TG ($t(22) = 4.38$, $p = 0.0002$) (Fig. 6B).

3.5. Twenty-four-hour ICV propranolol pretreatment did not alter harmine-induced tremor in TG or WT.

Twenty-four-hour ICV propranolol (versus vehicle) pretreatment had no effect on harmine-induced tremor in TG or in WT (Fig. 8A). Propranolol (versus vehicle) pretreatment had no effect on tremor, evaluated by $AUC_{0\text{-}90}$ (Fig. 8B) or on mean response of either TG or WT (Fig. 8C and 8D). Propranolol (versus vehicle) pretreatment trended towards increasing peak tremor in TG ($t(24) = 1.77$, $p = 0.0887$) (Fig. 7C), but not in WT (Fig. 7D).

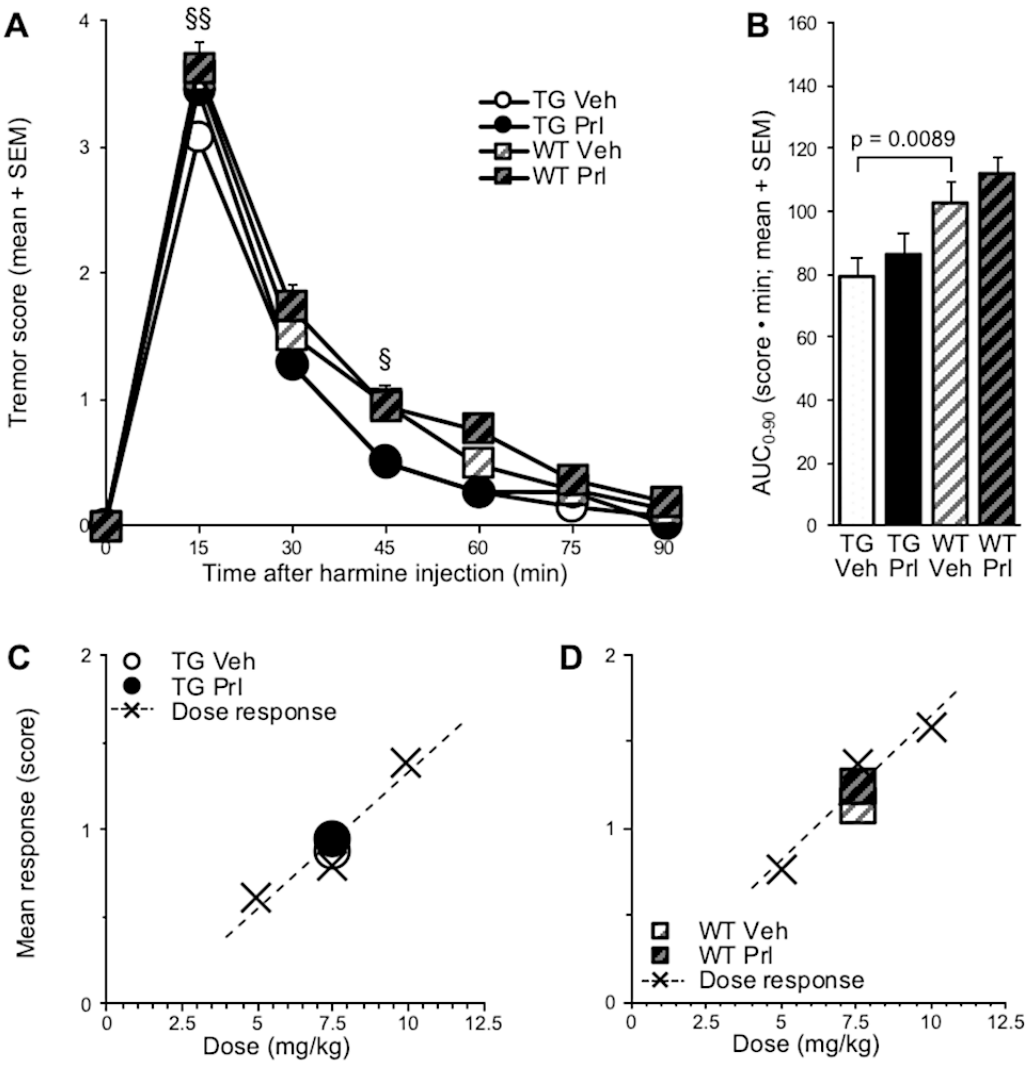

Figure 8. Twenty-four-hour ICV propranolol (versus vehicle) pretreatment did not alter harmine-induced tremor. A) Twenty-four hours after ICV propranolol or vehicle pretreatment, harmine treatment was administered, and tremor was scored for 90 minutes in TG (n=25) and WT (n=24). **B)** $AUC_{0\text{-}90}$ of propranolol or vehicle pretreated TG and WT. Mean response of propranolol or vehicle pretreated (**C**) TG and (**D**) WT; values are superimposed on dose response data from Fig 2C and 2D respectively. Vehicle pretreated WT versus TG: §$p < 0.05$, §§$p < 0.01$. Veh, vehicle; Prl, propranolol; SEM, standard error of the mean; AUC, area under the curve.

Tremor was more severe in vehicle-pretreated WT compared to TG (mouse line, $F(1,47) = 7.48$, $p = 0.0088$) (Fig. 8A). There was a main effect of mouse line on tremor, measured by $AUC_{0\text{-}90}$ ($F(1,47) = 15.5$, $p = 0.0003$), due to $AUC_{0\text{-}90}$ being greater in vehicle-pretreated WT compared to TG ($t(47) = 2.73$, $p = 0.0089$) (Fig. 8B).

3.6. Effect of ICV pretreatment on in vitro CYP2D enzymatic activity in brain and in liver

Four-hour ICV propranolol (versus vehicle) pretreatment yielded a non-significant decrease in *in vitro* brain dextrorphan formation in TG ($t(9) = 1.28$, $p = 0.2312$); removal of one outlier in the propranolol group revealed a significant difference ($t(8) = 2.32$, $p = 0.0488$) (Fig. 9A). There was no effect of four-hour ICV propranolol (versus vehicle) pretreatment in

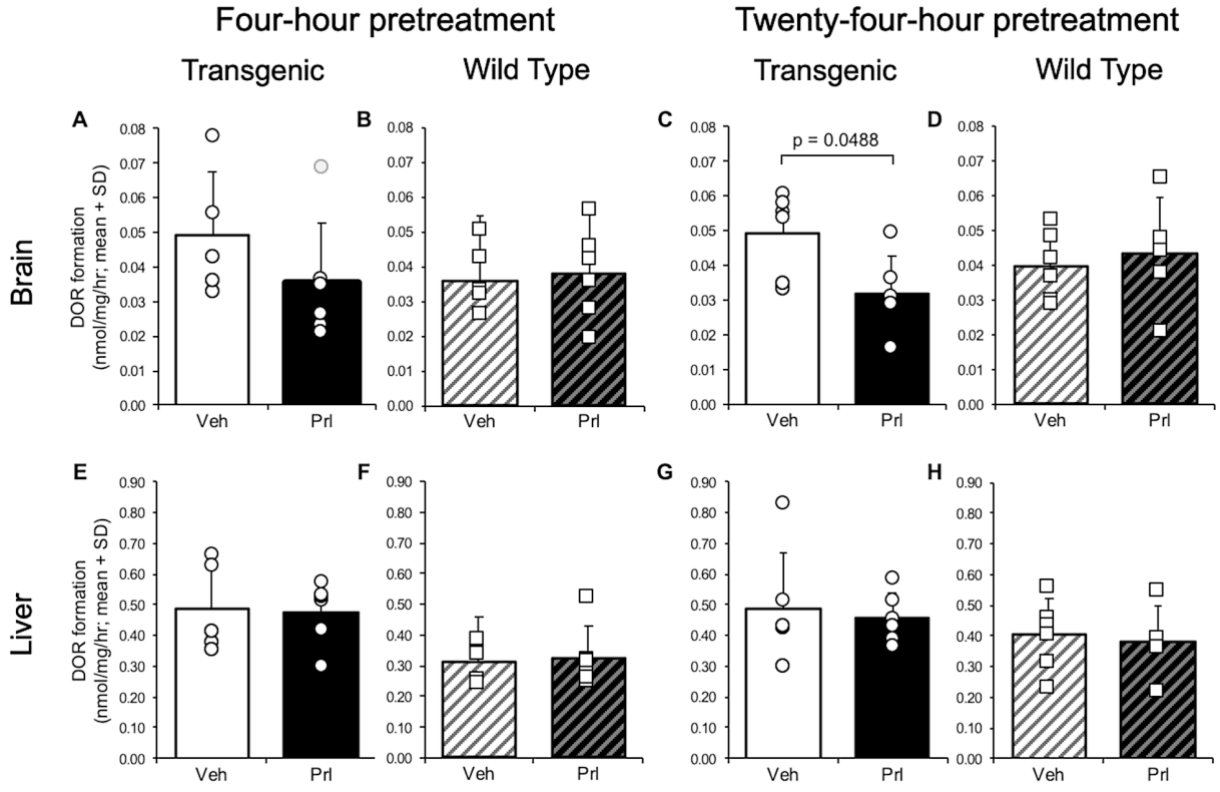

Figure 9. ICV propranolol (versus vehicle) pretreatment reduced in vitro brain CYP2D activity in TG. In vitro brain dextrorphan formation after four-hour ICV propranolol or vehicle pretreatment in (**A**) TG (outlier in grey) and (**B**) WT, and after twenty-four-hour ICV pretreatment in (**C**) TG and (**D**) WT. In vitro liver dextrorphan formation after four-hour ICV propranolol or vehicle pretreatment in (**E**) TG and (**F**) WT, and after twenty-four-hour ICV pretreatment in (**G**) TG and (**H**) WT. Data were normalized to the vehicle-pretreated TG group in the four-hour ICV pretreatment experiment. DOR, dextrorphan; Veh, vehicle; Prl, propranolol; SD, standard deviation

WT (Fig. 9B). Twenty-four-hour ICV propranolol (versus vehicle) pretreatment reduced *in vitro* brain dextrorphan formation in TG ($t(10) = 2.63$, $p = 0.0253$) (Fig. 9C), but not in WT (Fig. 9D). Taken together, the effects of the 4-hour and 24-hour pretreatments are consistent with ICV propranolol irreversibly inhibiting human CYP2D6 expressed in TG brain, but not irreversibly inhibiting mouse CYP2D in TG or WT brain. There was no effect of four- or twenty-four-hour ICV propranolol (versus vehicle) pretreatment on *in vitro* liver dextrorphan formation in TG or WT (Fig. 9E-H), consistent with ICV propranolol not crossing into the peripheral system in sufficient amounts to inhibit CYP2D6 in TG liver or mouse CYP2D in TG and WT liver.

4. Discussion

To study the role of CYP2D in general within the brain in harmine-induced hypothermia and tremor responses, a 4-hour pretreatment with ICV propranolol was given. This 4-hour ICV propranolol pretreatment, which irreversibly inhibits human CYP2D6 in TG brain and competitively inhibits mouse CYP2D in TG and WT brain, exacerbated harmine-induced hypothermia and tremor in TG and exacerbated harmine-induced hypothermia in WT. This indicates a role for CYP2D in general in the brain to alter this neurotoxicity. To then study the role of human CYP2D6 specifically within the brain in harmine-induced hypothermia and tremor responses, a 24-hour pretreatment with ICV propranolol was given. This 24-hour ICV propranolol pretreatment, which selectively and irreversibly inhibits human CYP2D6 in TG brain, exacerbated harmine-induced hypothermia in TG. This study is the first demonstration that human CYP2D6 specifically, expressed in mammalian brain, has sufficient activity *in vivo* to mitigate neurotoxin-induced response.

Beta-carbolines and their precursors are consumed in animal protein and some plant-derived foods (19, 20), as well as in alcoholic beverages and through tobacco smoke inhalation (20); beta-carbolines can also be formed endogenously (40, 41). Beta-carbolines can be neurotoxic *in vitro* and *in vivo* (42, 43). Many beta-carbolines are structurally similar to MPTP and can be bioactivated in the brain to N-methyl-beta-carbolinium cations (44), which are analogs of the Parkinsonism-inducing neurotoxic metabolite MPP^+ (10, 45). The CYP2D6-mediated detoxification of beta-carbolines could provide an alternative protective route in competition with a bioactivation pathway. Elevated exposure to beta-carbolines, either through consumption or endogenous production, may contribute to the etiology of some neurodegenerative disorders (21, 46). Harmine is a beta-carboline and a metabolite of harmane (47), and both are present in human tissue (41). Harmane levels are higher in those with Essential tremor (23) and Parkinson's disease (48). In animals, harmine causes hypothermia (24) and tremor (25). Human CYP2D6 and mouse CYP2D catalyze the O-demethylation of harmine to its non-hypothermic and non-tremorgenic metabolite harmol (7).

Harmine-induced hypothermia is centrally mediated, with some evidence for the involvement of a serotonergic pathway in the hypothalamus (24, 49). Many neurotoxins, including MPTP and chlorpyrifos, induce hypothermia via central mechanisms (50, 51). A 4-hour ICV propranolol pretreatment exacerbated harmine-induced hypothermia in TG and WT. Of note, this pretreatment had no effect on body temperature measured after IP treatment with distilled water, suggesting a lack of off-target effects of ICV propranolol pretreatment. This 4-hr pretreatment effect is consistent with irreversible inhibition of human CYP2D6 in TG brain and competitive inhibition of mouse CYP2D in TG and WT brain, whereby harmine inactivation to harmol was reduced in the brain, exacerbating harmine response in both TG and WT. This suggests that CYP2D isoforms in the brain metabolize

harmine, and that more of this metabolism confers protection against beta-carboline-induced neurotoxicity. Consistent with previous reports, hypothermia was less severe in TG compared to WT (28), owing to their expression of human CYP2D6 in addition to mouse CYP2D in both liver and brain, yielding more rapid inactivation of harmine to harmol (7). Pretreatment with ICV propranolol does not inhibit hepatic metabolism or alter peripheral drug levels (34), meaning ICV propranolol-pretreated TG (versus WT) presumably have more rapid harmine metabolism in the liver due to human CYP2D6 and mouse CYP2D activity in TG liver. Nevertheless, hypothermia response did not differ between propranolol-pretreated TG and WT, suggesting that harmine metabolism in the brain may be a more important determinant of hypothermia severity than metabolism in the liver.

A 24-hour ICV propranolol pretreatment irreversibly inhibits human CYP2D6 in TG brain and has no effect on mouse CYP2D in TG or WT brain. This pretreatment increased harmine-induced hypothermia in TG and had no effect in WT, used to control for the off-target effects of 24-hour ICV propranolol pretreatment. The impact of 24-hour pretreatment on TG (versus WT) is consistent with reduced CYP2D6-mediated harmine inactivation to harmol in TG brain exacerbating response, providing further evidence that harmine metabolism in the brain (versus the liver) is an important determinant of hypothermia severity. This suggests that human CYP2D6, expressed in TG brain, contributes significantly to harmine inactivation and is sufficient to mitigate this measure of beta-carboline-induced neurotoxicity.

Essential tremor is the most common neurodegenerative movement disorder, affecting approximately 5% of those over the age of 65 (52). Beta-carbolines have been causally implicated in Essential tremor etiology (53), as levels in blood (54) and in brain (23)

were elevated in those with Essential tremor compared to healthy controls. Furthermore, among a group of individuals with Essential tremor, beta-carboline levels in blood were negatively correlated with cerebellar N-acetylaspartate/total creatine (NAA/tCR), which is a measure of metabolic function; therefore, higher beta-carboline levels were associated with lower metabolic function, indicating more degeneration in the cerebellum (21). In fact, cerebellar degeneration is a common feature of Essential tremor (55) and of beta-carboline administration in animal models (56). Many beta-carbolines also induce a tremor response in animals that shares qualitative characteristics with the tremor in humans (26). Beta-carbolines, including harmine and its congener harmaline, are commonly used in rodents to model Essential tremor (53, 57).

The 4-hour ICV propranolol pretreatment, which irreversibly inhibits human CYP2D6 and competitively inhibits mouse CYP2D in TG brain, increased harmine-induced tremor in TG. This is consistent with inhibition of CYP2D6- and CYP2D-mediated harmine inactivation in the brain exacerbating harmine response. Contrary to our hypothesis, 4-hour pretreatment did not exacerbate tremor in WT. In our dose response experiments, a harmine dose of 7.5 mg/kg IP (used in this experiment) produced a near-maximal tremor response in WT, indicating a potential ceiling effect. Additionally, the observational tremor scale may lack the sensitivity to detect subtle differences in tremor severity. Therefore, it may not have been possible to detect an increase in tremor severity following 4-hour pretreatment in WT, especially if the effect was not robust.

Following a 24-hour ICV propranolol pretreatment, which selectively and irreversibly inhibits human CYP2D6 in TG brain, we observed an increase in peak tremor that trended towards significance in TG, suggesting that inhibiting CYP2D6-mediated harmine

inactivation to harmol in the brain may enhance tremor response. Although we did not observe as large of an effect on tremor of the 24-hour pretreatment compared to the 4-hour pretreatment, this is consistent with a lesser effect of inhibiting human CYP2D6 but not mouse CYP2D in TG brain, due to the uninhibited mouse CYP2D in TG brain metabolizing harmine. Nevertheless, the smaller effect of 24-hour pretreatment on harmine-induced tremor response contrasts with the robust effect on harmine-induced hypothermia. The difference between hypothermia and tremor may be due to these responses being generated via discrete central mechanisms, which may be differentially impacted by local CYP2D and/or CYP2D6 metabolism in the brain. Harmine induces synchronous burst firing of neurons originating in the inferior olive of the medulla oblongata, which then project to the cerebellum where tremor is generated by activation of Purkinje cells and Deep Cerebellar Nuclei (26, 58). Although CYP2D6 is expressed in TG cerebellum (29), the inferior olive (where burst firing originates) is proximal to the area postrema, which lacks the blood-brain barrier (59). Thus, peripheral drug levels and hepatic metabolism, which are not affected by pretreatment (34), may have a greater impact on harmine-induced tremor severity, in contrast with the greater impact of brain drug levels and brain metabolism on harmine-induced hypothermia response.

CYP2D6 metabolism constitutes an inactivation pathway for several neurotoxins, suggesting a protective role for CYP2D6 for these toxins (5-7). Genetic *CYP2D6* poor metabolizers are at higher risk for developing Parkinson's disease (12), and this risk is increased further by lifetime exposure to environmental toxins, which are causally implicated in idiopathic Parkinson's disease, as well as being CYP2D6 substrates (60, 61). This suggests a gene-environment interaction whereby CYP2D6-mediated inactivation following xenobiotic neurotoxin exposure reduces neurotoxicity and subsequent neurodegeneration.

Human CYP2D6 expressed in the TG brain was sufficient to mitigate specific neurotoxicity-related responses to harmine, despite harmine being administered peripherally and undergoing first-pass metabolism. This suggests that CYP2D6 expressed in the human brain may play a role in protecting against beta-carboline-induced neurotoxicity, even in the case of peripheral exposure, such as that occurring through consumption of certain foods and beverages. The large interindividual variation in CYP2D6 levels in the human brain (33) may influence local metabolism of beta-carbolines and other neurotoxins, which could then impact the severity of neurotoxicity and resultant neurodegeneration. CYP2D6 is expressed in brain regions that support a role in protecting against Essential tremor (cerebellum) and Parkinson's disease (substantia nigra and caudate-putamen) (33, 62). Despite CYP2D in the liver being uninducible, CYP2D in the brain can be induced by xenobiotic exposure. For example, repeated daily nicotine administration increases CYP2D in the brain (but not in the liver) of mice (63), rats (18), and non-human primates (17). Consistent with this, human cigarette smokers have more CYP2D6 in the brain compared with non-smoking controls (17). Furthermore, CYP2D6 levels were higher in the cerebellum and substantia nigra (17); neurodegeneration in these regions is associated with Essential tremor (55) and Parkinson's disease (64). Cigarette smoking is associated with decreased risk for Essential tremor (65) and Parkinson's disease (66), suggesting that the neuroprotective effect of smoking may be due, at least in part, to higher CYP2D6 in the brain increasing local neurotoxin inactivation. This could confer protection against neurotoxicity and the subsequent neurodegeneration characteristic of these neurodegenerative disorders.

CYP2D6 metabolism in the human brain is of increasing interest. CYP2D6 is expressed in neurons and glia within discrete brain regions (13, 33), and its substrates include centrally acting endogenous compounds. CYP2D6 was shown *in vitro* to catalyze the 6β-,

21-, and 16α-hydroxylation of progesterone (67), as well as the formation of dopamine from tyramine (68) and serotonin from 5-methoxytryptamine, with evidence for the latter occurring *in vivo* (69). Variation in CYP2D6 in the brain may impact neurological, cognitive, and psychosocial function, as evidenced by their associations with *CYP2D6* genotype and central mechanisms. For example, the genotype-derived CYP2D6 activity score was found to be inversely related with cerebral blood flow in the thalamus, where CYP2D6 is expressed (13, 70). Genetic *CYP2D6* poor metabolizers scored higher on measures of impulsiveness, perfectionism, and sustained attention, and they scored lower on general psychopathology, as assessed in a series of personality and cognitive tests (71). Genetic *CYP2D6* extensive and ultra-rapid metabolizers were also more frequent among patients with eating disorders compared to healthy controls (72). Thus, the ability to selectively manipulate human CYP2D6 in a mouse brain and use WT as controls, as applied herein, provides a novel tool to investigate these and other endogenous functions of CYP2D6 in the brain, as well as its impact on various cognitive and behavioral outcomes.

5. Conclusions

This novel approach was developed to demonstrate that CYP2D6 is functional *in vivo* and can alter brain drug and metabolite levels, as well as drug response (34). Here, we show for the first time that human CYP2D6 in brain can alter neurotoxin response following peripheral beta-carboline administration; specifically, CYP2D6 in brain mitigated harmine-induced neurotoxicity. In conclusion, CYP2D6 in the human brain may confer some neuroprotection against beta-carboline-induced neurotoxicity. This represents a potential novel source of variation in susceptibility to neurotoxicity and to neurodegenerative disorders that are associated with elevated neurotoxin (e.g. beta-carboline) exposure, including

Essential tremor and Parkinson's disease. More broadly, this model can be used to test novel neurotoxins and centrally acting drugs for the impact of CYP2D, and human CYP2D6 specifically, in the brain on resulting behaviors *in vivo*.

Declarations

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Author Contributions

M.R.S. and C.T. contributed equally as first authors on this paper. M.R.S. and C.T. performed the experiments and analyzed the data. F.B.W. maintained the transgenic mouse breeding colony and assisted with surgeries. M.R.S., C.T., S.M., and R.F.T contributed to the study design. M.R.S., C.T., S.M., F.J.G., and R.F.T., contributed to the manuscript writing. All authors approved the final version of this paper.

Compliance with ethical standards

Conflicts of interest: R.F.T. has consulted for Quinn Emanuel and Ethismos Research Inc. All other authors declare no conflict of interest.

Ethics approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Experiments were performed in accordance with the NIH guidelines for the care and use of laboratory animals, and with approval of the University of Toronto Animal Care Committee.

Data and Material Availability

Supporting data and material can be found in the additional files and can be requested from the corresponding author.

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