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Centrally administered CYP2D inhibitors increase oral tramadol analgesia in rats

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Abstract

Cytochrome P450 2D (CYP2D) mediates the activation and inactivation of several classes of psychoactive drugs, including opioids, which can alter drug response. Tramadol is a synthetic opioid with analgesic activity of its own as well as being metabolically activated by CYP2D to O-desmethytramadol (ODMT) an opioid receptor agonist. We investigated the impact of brain CYP2D metabolism on central tramadol and ODSMT levels, and resulting analgesic response after oral tramadol administration in rats. CYP2D inhibitors propranolol and propafenone were administered intracerebroventricularly prior to oral tramadol administration and analgesia was measured by tail-flick latency. Drug levels of tramadol and its metabolites, ODSMT and N-desmethytramadol, were assessed in plasma and in brain by microdialysis using LC-ESI-MS/MS. Inhibiting brain CYP2D with propafenone pretreatment increased analgesia after oral tramadol administration (ANOVA $p=0.02$), resulting in a 1.5-fold increase in area under the analgesia-time curve (AUC_{0-60} , $p<0.01$). This effect was associated with changes in the brain levels of tramadol and its metabolites consistent with brain CYP2D inhibition. In conclusion, under oral tramadol dosing pretreatment with a central administration of the CYP2D inhibitor propafenone increased analgesia (without altering plasma drug or metabolite levels), indicating that tramadol itself (and activity of CYP2D within the brain) contributed to analgesia.

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⁶Competing Interests

R.F.T. has consulted for Quinn Emanuel and Ethismos on unrelated topics. All other authors declare no competing interests.

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Keywords

Brain; cytochrome P450; metabolism; tramadol; opioids; analgesia

1 Introduction

Tramadol is a synthetic centrally acting opioid analgesic effective in the treatment of mild-to-moderate pain (e.g. neuropathic and osteoarthritis-related pain) (Cepeda et al., 2006, Subedi et al., 2019). Tramadol is atypical relative to common opioid analgesics (e.g. codeine and oxycodone) in that it induces analgesia through serotonin and norepinephrine reuptake inhibition in addition to μ -opioid receptor activation by a tramadol metabolite (Raffa et al., 1992). Tramadol is extensively metabolized to O-desmethyltramadol (ODSMT) and N-desmethyltramadol (NDSMT) mainly by the cytochrome P450 (CYP) enzymes CYP2D6 (herein referred for simplicity to as CYP2D for rat and all other non-human species) and CYP3A4, respectively (Wu et al., 2002). ODSMT is considered primarily responsible for tramadol analgesia due to its potent agonist activity at the μ -opioid receptor relative to tramadol, while tramadol itself displays serotonin and norepinephrine reuptake inhibitory activity which may be responsible for monoamine-related side effects (Bannister et al., 2009, Park et al., 2014, Zebala et al., 2019). For this reason, there were recent efforts to introduce ODSMT alone (i.e. “Desmetramadol”) as an analgesic alternative to tramadol (Zebala et al., 2019). NDSMT, as well as additional non-CYP-mediated tramadol metabolites formed by sulfation and glucuronidation pathways, does not contribute to tramadol analgesia (Nobilis et al., 2002).

While the analgesic activity of tramadol is believed to be primarily due to activation of the μ -opioid receptor by the CYP2D metabolite ODSMT, the contribution of CYP2D metabolism to tramadol analgesia is unclear. If tramadol were a prodrug requiring enzyme activation for effect (e.g. codeine), administration by a route that enhances CYP2D formation of ODSMT should increase tramadol analgesia (Zebala et al., 2019). In agreement with this, intravenous tramadol was more potent than epidural tramadol (i.e. centrally administered) when compared to morphine in patients treated for postoperative pain (Lee et al., 1993). However, when compared with oral tramadol (30% of a tramadol dose undergoes first-pass metabolism), intravenous tramadol remained more potent in postoperative pain prevention (Nobilis et al., 2002, Ong et al., 2005). Likewise, individuals given the CYP2D6 inhibitor quinidine showed no decrease in oral tramadol analgesia (Collart et al., 1993). In *CYP2D6* poor metabolizers given oral tramadol, serum ODSMT was barely detectable, as expected, but analgesia was still observed (Poulsen et al., 1996). Together, this suggests a more complex relationship between CYP2D-mediated metabolism of tramadol and analgesia than previously thought.

CYP enzymes are expressed and active in the brain and are regulated independently of hepatic CYPs (McMillan and Tyndale, 2018). Modifying CYP2D activity selectively in the rat brain, using a centrally administered CYP2D inhibitor and/or a brain CYP2D inducer, alters brain drug and metabolite levels, resulting in the alteration in behavioral response of centrally-acting CYP2D substrate drugs, such as codeine and oxycodone (Zhou et al., 2013,

McMillan and Tyndale, 2015, 2017, Miksys et al., 2017, McMillan et al., 2019). Applying paradigms developed by our group, Wang et al. showed, using intraperitoneal (i.p.) tramadol administration, that inhibiting brain CYP2D prolonged the elimination $t_{1/2}$ of tramadol in the brain and decreased tramadol analgesia whereas inducing brain CYP2D elevated brain ODSMT levels and resulting analgesia, consistent with a primary role for ODSMT in tramadol analgesia (Wang et al., 2015). However, 1) tramadol was administered i.p. rather than the more clinically relevant oral route of administration, and 2) the impact of brain CYP2D inhibition on central ODSMT levels and resulting tramadol analgesia was modest (Wang et al., 2015).

In the current study, we sought to study the impact of CYP2D-mediated metabolism in the brain on the brain levels of tramadol and its metabolites and the resulting analgesia when tramadol was given by oral gavage (p.o.). We used two structurally and mechanistically distinct CYP2D inhibitors given intracerebroventricularly (i.c.v.). Propranolol is a CYP2D mechanism-based inhibitor in rats (Narimatsu et al., 2001). Propranolol 24 hr pretreatment i.c.v. inhibits brain, but not liver, CYP2D activity as measured *in vivo* by brain microdialysis and plasma drug levels, and *ex vivo* by enzyme activity. This approach has been developed as a paradigm for testing the role of brain CYP2D on systemic CYP2D-substrate administration (Zhou et al., 2013, McMillan and Tyndale, 2015, Miksys et al., 2017, McMillan et al., 2019). Inhibition of CYP2D in brain by propranolol pretreatment decreased codeine induced analgesia and increased oxycodone induced analgesia, when the opioid was tested subcutaneously (s.c.), i.p. and/or p.o. (Zhou et al., 2013, McMillan and Tyndale, 2015, McMillan et al., 2019). Propafenone is a CYP2D competitive-inhibitor that, when given i.c.v., decreased brain morphine levels from s.c. administered codeine and resulting codeine analgesia 30 min after pretreatment, indicating of brain CYP2D inhibition (Xu et al., 1995, Zhou et al., 2013). This i.c.v. propafenone pretreatment paradigm has been used and compared to i.c.v. propranolol pretreatment showing a similar reduction in s.c. and i.p. codeine analgesia, and similar increase in p.o. oxycodone analgesia (Zhou et al., 2013, McMillan and Tyndale, 2015, McMillan et al., 2019). Our objectives in this study were to 1) determine an equivalent analgesic dose of tramadol p.o. to the previously used i.p. paradigm (Wang et al., 2015) and compare this to other opioids, 2) determine systemic tramadol and metabolite levels to ensure the isolated impact of our distinct i.c.v. pretreatments on brain CYP enzyme activity, then 3) determine the functional impact of i.c.v. pretreatment with CYP2D inhibitors on p.o. tramadol analgesic response and assess the levels of parent and metabolites through *in vivo* microdialysis. Together, this work explores the importance of route of administration as a consideration in drug metabolism and response and expands the role for brain CYP metabolism on orally administered drug metabolism and response.

2 Materials and Methods

2.1 Animals

Adult male Wistar rats (Charles River Laboratories, Saint-Constant, Canada) were housed 1–3 a cage and maintained between 300–500 g in weight by food restriction. Only male rats were used in this study as previous studies optimizing the pretreatment paradigms were performed in male rats; research into sex differences in brain CYP2D activity *in vivo* and

functional influences of this is currently underway. Rats were maintained on a 12-hour light/dark cycle with experimentation occurring during the light cycle. Stress-related confounders were reduced through acclimation of animals to handling, equipment, and rooms prior to each experiment. All procedures were conducted in accordance with the Canadian Council on Animal Care guidelines for the care and use of laboratory animals and approved by the Animal Care Committee at the University of Toronto.

2.2 Drug Treatment

Tramadol hydrochloride (Sigma-Aldrich, Oakville, Canada) was dissolved in dH₂O and administered by intraperitoneal injection (i.p.) or oral gavage (p.o.) in 1 ml volume/kg. Selection of the oral dosing (in mg/kg) is outlined in Figure 1. Propranolol hydrochloride (Sigma-Aldrich) was dissolved in 20% 2-hydroxypropyl- β -cyclodextrin (Sigma-Aldrich) and injected as 20 μ g base in 4 μ l through intracerebroventricular (i.c.v.) infusion probes (2 mm, MD-2258, Bioanalytical Systems, Inc. [BASi], West Lafayette, IN, USA). Propafenone hydrochloride (Sigma-Aldrich) was dissolved in the same vehicle as propranolol and injected as 40 μ g base in 4 μ l through i.c.v. infusion probes (BASi). As previously mentioned, propranolol is a rat CYP2D mechanism-based inhibitor while propafenone is a rat CYP2D competitive-inhibitor (Xu et al., 1995, Narimatsu et al., 2001). Propranolol or propafenone i.c.v. injections have been previously used as described and shown to inhibit rat brain, but not liver, CYP2D enzyme activity 24 hr or 30 min after pretreatment, respectively (Zhou et al., 2013, McMillan and Tyndale, 2015, Miksys et al., 2017, McMillan et al., 2019).

2.3 Tail-flick Latency

Antinociception (denoted here as analgesia) was measured as the tail-flick latency (TFL) using a tail-flick meter (Columbus Instruments, Columbus, OH, USA). TFL was recorded as the time from the onset of a thermal stimulus to the withdrawal of the tail, as previously described (Le Bars et al., 2001, McMillan and Tyndale, 2015). The thermal strength of the tail-flick meter was adjusted prior to experimentation to obtain baseline TFLs of approximately 3–4 s. A cut-off of 12 s was used to minimize tissue damage and was therefore considered the maximal TFL. 2–3 baseline TFL measurements were taken prior to i.c.v. pretreatment and prior to tramadol administration. For full analgesia-time curves, analgesia was assessed over time with a focus on the first 60 min after tramadol administration to accurately assess peak analgesia (TFL timepoints = 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, and 90 min). Analgesia was displayed throughout as the percentage of maximal possible effect (%MPE = [post-injection latency – baseline latency] / [maximum cut-off – baseline latency] $\times$ 100 %).

2.4 Cannulation surgery and microdialysis procedure

Rats were anesthetized with isoflurane and injected with the analgesic meloxicam (2 mg/kg s.c.). Rats were surgically implanted with microdialysis guide cannulae (MD-2257, BASi) into the right lateral ventricles (Bregma coordinates: anteroposterior: –0.9 mm; lateral: –1.4 mm; dorsoventral, –3.6), as previously described (McMillan et al., 2019). Meloxicam was given daily (2 mg/kg s.c.) for 48 hr post-surgery and animals had one week of recovery before experimentation; they were housed singly to avoid headcap misplacement.

Microdialysis probes (2 mm, MD-2201, BASi) were inserted into the microdialysis guide-cannulae and animals were allowed to habituate for at least 10 minutes while perfusion medium (Ringer's solution; 147 mM Na⁺, 2 mM Ca²⁺, 4 mM-K⁺, 155 mM Cl⁻, pH 7.4) was circulated at a flow rate of 2 µl/min. Baseline dialysate samples were collected on ice in 3 × 10 min time bins prior to i.c.v. propafenone (30 min total, baseline 1), 3 × 10 min time bins following propafenone and prior to p.o. tramadol (30 min total, baseline 2), and 12 × 10 min time bins following tramadol gavage (120 min total). The timing of analyte levels was adjusted by 10 min to compensate for the 20 µl dead volume between the microdialysis probe and the sample collection outlet. Tramadol, ODSMT, and NDSMT concentrations for each microdialysis collection bin were measured by LC-MS/MS. Antinociception was measured by TFL at 30 min after tramadol injection, followed by a saphenous vein blood draw for serum drug levels.

2.5 LC-MS/MS assay and sample preparation

Tramadol, ODSMT, NDSMT, and their isotopomers, tramadol-D₃-¹³C, O-desmethyltramadol-D₆ and N-desmethyltramadol-D₃ were analyzed by LC-ESI-MS/MS assay adapted from previously published methods (Verplaetse and Tytgat, 2012, Tanaka et al., 2016, Liu et al., 2017). Agilent 1260 LC system, coupled with Agilent 6430 Triple Quadrupole system (Agilent Technologies) was used. Analytes were separated by Agilent C18 column (Zorbax SB-C18, 5µm 2.1 × 150 mm) at 35°C, using gradient elution of A (water + 0.1% formic acid) and B (methanol + 0.1% formic acid) at 0.25 ml/min; the following order was used from 35% to 50% B in 0.5 min, 0.5 min of 50% B, 50% to 65% of B in 0.6 min, 0.4 min of 56% B, followed by 3 min column reequilibration. Mass spectrometer was operated with multiple-ion monitoring under positive-ion mode, divided into three time segments, in the first segment, from 2 to 3.2 min one transitions was monitored for ODSMT, 250.2 → 58.1 (CID = 14 V; fragmentor voltage= 88 V; dwell time 200 ms), and in the second segment, from 3.2 to 5.4 min, one transitions for tramadol, 264.2 → 58.1 (CID = 14 V; fragmentor voltage= 91 V; dwell time 200 ms) and NDSMT, 250.2 → 44.1 (CID = 14 V; fragmentor voltage= 88 V; dwell time 200 ms); mobile phase was directed to waste otherwise. Gas temperature, flow and pressure were set at 350°C, 11 l/min and 15 psi, respectively, and the capillary voltages were 2.5 kV.

The assay linearity was checked between 0.25 to 1000 pg on column. The extraction efficiency and matrix effect were measured for tramadol, ODSMT and NDSMT in dialysate and serum. The assay was validated for precision and accuracy of the assay by analyzing four replicates at four different concentrations, 0.05, 1, 20 and 100 ng/ml for dialysate and 0.25, 7.5, 75 and 750 ng/ml for serum.

Dialysate samples were spiked with the internal standards (2 ng/ml final concentration), followed by centrifugation at 15,000 rpm for 5 min, and the supernatants were injected to LC-MS/MS. Serum samples were diluted 12 times with control serum and spiked with internal standards (2.5 ng/ml final concentration). Serum proteins were precipitated from diluted spiked samples by the addition of methanol (66% v/v final concentration) and incubating at 4°C for 15 min, followed by centrifugation at 15,000 rpm for 5 min. The

supernatant was diluted by the addition of Milli-Q water (50% methanol final concentration) and injected to LC-MS/MS.

2.6 Microdialysis probe recovery

The recoveries of the microdialysis probes (2 mm, MD-2201, BASi) were assessed in vivo and in vitro for tramadol, ODSMT and NDSMT. For the in vitro recovery, the microdialysis probes were incubated in a well-stirred artificial cerebrospinal fluid solution of tramadol (50 or 400 ng/ml), ODSMT (1 or 40 ng/ml), and NDSMT (2 or 300 ng/ml) at 37°C. Ringer's solution was perfused through the microdialysis probe and the dialysate was collected every 15 min to get 11 microdialysate samples. The concentration (C) of tramadol and its metabolites in the dialysate was measured by LC-MS/MS to calculate the %gain = $C_{\text{Dialysate}}/C_{\text{Nominal}} \times 100$. For the in vivo recovery, Ringer's solution containing 20 ng/ml of tramadol, ODSMT, and NDSMT was perfused at 2 µl/min through the microdialysis probe inserted in control animals, and the dialysate was collected every 15 min to get 11 microdialysate samples. The concentration (C) of tramadol and its metabolites in the dialysate and perfusate was measured by LC-MS/MS to calculate the %loss = $(C_{\text{Perfusate}} - C_{\text{Dialysate}})/C_{\text{Perfusate}} \times 100$. The in vitro and in vivo probe recovery data is displayed in Supplementary Figure 1.

2.7 Statistical Analyses

Data were analyzed with GraphPad Prism v.8.2.1 (GraphPad Software Inc., La Jolla, CA, USA), by two-way ANOVA followed by either independent samples *t*-tests (AUC and single analgesia time-points) or Tukey's *post hoc* testing adjusted for multiple comparisons (brain drug levels in microdialysis bins or %MPE at individual time points). Animal group sizes were powered to detect differences between pretreatment groups using *n*=5/group; additional animals were included in some experiments to account for loss of surgical headcap during i.c.v. pretreatment. Repeated measures were used in within-animal experiments.

3 Results

3.1 Dose optimization for tramadol-induced analgesia from oral gavage

In order to assess the impact of changing route of administration on tramadol analgesia, we determined the tramadol p.o. dose equivalent to 40 mg/kg tramadol i.p.; this i.p. dose was used previously to assess the impact of brain CYP2D metabolism on tramadol analgesia (Wang et al., 2015). Compared to previously published data, 40 mg/kg tramadol i.p. produced similar peak analgesia (approximately 52% published vs. 65% MPE) and time to peak analgesia (45 published vs. 50 min post-injection) and greater overall analgesic response (1740 published vs. 2840 %MPE*min AUC 0–60 min) (Wang et al., 2015). Under the same conditions, we tried 5 different tramadol doses p.o. (60–120 mg/kg) to obtain 1) a similar analgesic response with tramadol p.o. administration as was observed with 40 mg/kg i.p. and 2) to measure peak tramadol response, AUC, and maintain a sub-maximal peak capable of detecting an anticipated decrease in drug response. A tramadol p.o. dose of 100 mg/kg provided a similar peak analgesia to 40 mg/kg tramadol i.p. (65% vs. 74% MPE; Figure 1a and 1b), and was used in all subsequent studies. Additional dose-response comparisons between tramadol and other opioids previously tested, including p.o. vs. i.p.

morphine and codeine (McMillan and Tyndale, 2015, 2017, McMillan et al., 2019) are shown in Supplementary Figure 2.

3.2 Impact of i.c.v. propranolol and propafenone pretreatment on serum tramadol and metabolite levels after p.o. drug administration

Compared with vehicle, propranolol pretreatment appeared to increase serum parent tramadol ($p=0.18$), and its metabolites, ODSMT ($p=0.17$) and NDSMT ($p=0.25$) levels after tramadol p.o. (Figure 2a-c). This occurred despite not altering hepatic CYP2D metabolism or peripheral drug levels when used previously (confirmed through *in vivo* drug level analysis, and *ex vivo* metabolism assays) (Zhou et al., 2013, McMillan and Tyndale, 2015, Miksys et al., 2017, McMillan et al., 2019). The apparent increase in serum tramadol and the CYP2D metabolic ODSMT was not consistent with an inhibition of hepatic CYP2D, where a decrease in ODSMT would be expected. Furthermore, the variability in serum drug and metabolite levels in the propranolol pretreatment group, as displayed by large standard deviation, may be indicative of a potential drug-interaction with propranolol and tramadol beyond that involving CYP metabolism thereby making further interpretation of CNS metabolism data impossible. In addition, the impact of i.c.v. propranolol pretreatment on brain tramadol and metabolite levels was assessed in a pilot study using *in vivo* microdialysis. Consistent with the serum data, 24 hr i.c.v. propranolol pretreatment increased brain levels of tramadol and its metabolites, ODSMT and NDSMT, resulting in an increase in analgesia after tramadol p.o. (Supplementary Figure 3). NDSMT levels were assessed throughout as a control to ensure the pretreatments used to modify brain CYP2D activity were selectively affecting CYP2D metabolism alone. In contrast, compared with vehicle, i.c.v. propafenone 30 min pretreatment did not alter serum tramadol ($p=0.64$), ODSMT ($p=0.54$), or NDSMT ($p=0.91$) levels after tramadol p.o. (Figure 2d-2f) as expected. As propafenone had no impact on the serum drug levels, it was used going forward as an acute competitive inhibitor of CYP2D.

3.3 Effect of acute i.c.v. pretreatment with competitive CYP2D inhibitor propafenone on tramadol p.o. analgesia

The impact of i.c.v. propafenone on tramadol-induced analgesia was assessed after p.o. tramadol administration. Compared to vehicle, propafenone pretreatment increased tramadol-induced analgesia (0–60 min $p=0.02$; Figure 3a), resulting in a 2.4-fold increase in peak analgesia and 1.5-fold increase in the area under the analgesic-time curve from 0–60 min (AUC 0–60 min $p<0.01$; Figure 3b) after tramadol gavage. A within-animal study design was used where animals received i.c.v. vehicle or propafenone pretreatment in randomized order with a two-week washout between tests; this illustrated the consistency of impact of the propafenone inhibitor pretreatment on individual animal analgesic AUCs (0–60 min) (Figure 3b). There was no difference between baseline TFLs measured before and after i.c.v. vehicle pretreatment (3.28 ± 0.59 vs. 3.39 ± 1.63 mean $\pm$ SD, $p=0.78$), before and after i.c.v. propafenone pretreatment (3.64 ± 1.08 vs. 3.75 ± 1.64 mean $\pm$ SD, $p=0.75$), and between animals after i.c.v. vehicle and propafenone pretreatments (3.39 ± 1.63 vs. 3.75 ± 1.64 mean $\pm$ SD, $p=0.64$).

3.4 Microdialysis brain levels of tramadol and metabolites after acute inhibition with propafenone; analgesia during microdialysis and brain homogenate levels after sacrifice

To elucidate how the increase in tramadol-induced analgesia was associated with changes in brain parent tramadol and metabolite levels over time, i.c.v. *in vivo* brain microdialysis was used to assess free brain drug levels in live animals. Baseline analyte measurements taken 30 mins prior to i.c.v. vehicle or propafenone and for 30 mins after pretreatment indicated that there were no detectable analyte levels prior to p.o. tramadol administration (data not shown). Compared with vehicle, propafenone i.c.v. pretreatment modestly but non-significantly increased brain tramadol levels ($p=0.27$; Figure 4a) and decreased brain ODSMT levels ($p=0.62$; Figure 4b) and brain NDSMT levels ($p=0.60$; Figure 4d) after p.o. tramadol. Taken together, the pattern of increased tramadol and decreased ODSMT brain levels suggests an impact of propafenone on brain CYP2D metabolism, while supporting a role for the parent compound tramadol in mediating tramadol analgesia. Although not powered to examine analgesia as we were in Figure 3, a similar trend was observed, where compared to vehicle, propafenone pretreatment increased tramadol analgesia in 5 of 6 animals (tested in animal, $p=0.09$; Figure 4d). As previously observed (Figure 2d-f) there was no impact of propafenone pretreatment on serum drug levels at 30 min ($p>0.8$; Figure 4e).

4 Discussion

In the current study, we investigated the impact of central administration of CYP2D inhibitors on oral tramadol analgesia, a route used in clinical practice. Previously Wang et al. showed that the injection of propranolol into the brain led to a small decrease in tramadol analgesia after 40 mg/kg i.p. tramadol with little effect on cerebrospinal fluid levels of tramadol or ODSMT (Wang et al., 2015). Our first step was to find a tramadol p.o. dose (i.e. 100 mg/kg) that was pharmacodynamically (i.e. analgesia) equivalent to the previously studied 40 mg/kg i.p. tramadol, allowing differences in the effect of brain CYP2D inhibition on tramadol analgesia to be attributed to pharmacokinetic factors. We have shown that opioids, (i.e. morphine, codeine or tramadol), show 2.5 – 10-fold decreases in potency when moving from i.p. to p.o. route of administration indicating these are not pharmacokinetically and/or pharmacodynamically identical routes of administration. Next, we examined the impact of CYP2D inhibition in the brain using two different chemical inhibitors, propranolol and propafenone. Propranolol and propafenone have been shown to selectively inhibit brain CYP2D without affecting systemic/hepatic metabolism using the same procedure followed in the current study. However, propranolol given i.c.v. 24 hr before tramadol p.o. resulted in an increase in tramadol and its metabolites in serum, and as a result, in the brain; this defeated our ability to use i.c.v. propranolol to examine the role of brain CYP2D on p.o. tramadol metabolism and analgesia. For propafenone, tramadol analgesia was increased after propafenone treatment, without any alteration in the systemic concentration of tramadol or its metabolites. The increase in tramadol analgesia following pretreatment with a CYP2D inhibitor was consistent with expected direction of change in the brain level of tramadol and ODSMT (increased and decreased respectively). Together, these findings suggest a role for CYP2D in brain on the metabolism of tramadol, and resulting decreased analgesia, using a

clinically relevant oral route of administration and further suggest that tramadol plays a role in analgesia following oral dosing.

Tramadol has an established wide variability in pharmacokinetic and pharmacodynamic properties, due largely in part to 1) chirality of tramadol and metabolites and 2) metabolism by a highly genetically polymorphic enzyme, *CYP2D6*. Tramadol is optically active and exists as a racemate with two active enantiomers, (+) and (–) enantiomers, each metabolized to the corresponding enantiomer of ODSMT and NDSMT (Poulsen et al., 1996, Subedi et al., 2019). (+)-ODSMT is considered the primary contributor to tramadol analgesia due to its affinity at the μ -opioid receptor (Gillen et al., 2000). However, findings on the contribution of *CYP2D6* genetic polymorphism to tramadol response in humans does support this contention; as mentioned, inhibition of *CYP2D6* by quinidine did not decrease oral tramadol analgesia in humans while *CYP2D6* poor metabolizers, with little-to-no production of ODSMT, continue to exhibit tramadol-induced analgesia (Collart et al., 1993, Poulsen et al., 1996). While (+)-ODSMT mainly acts a μ -opioid receptor agonist, (+)-tramadol and (–)-tramadol inhibit serotonin and norepinephrine reuptake, respectively (Raffa et al., 1993, Gillen et al., 2000). Serotonin and norepinephrine are endogenous substrates in descending pain modulatory pathways and are often indicated in analgesic combination therapies (Shen et al., 2013). Norepinephrine reuptake inhibitors were effectively antinociceptive in reducing pain-responses in rat models of inflammatory (formalin paw injection) and neuropathic (chronic constriction injury, sciatic nerve ligation) pain, while serotonin reuptake inhibitors were antinociceptive to inflammatory pain alone (Mochizucki, 2004). Thus, under some circumstances the parent compound tramadol, despite being a less potent μ -opioid receptor activator than ODSMT, could contribute to analgesia, consistent with our findings. Ultimately, the complementary and synergistic actions of (+)-ODSMT, (+)-tramadol, and (–)-tramadol may combine to confer a unique pharmacodynamic profile of action (Kanaan et al., 2009) which in turn may be altered by variation in dosing route and metabolism. In a rat formalin model of nociception, inhibition of both serotonin and norepinephrine transporters was required for opioid-mediated antinociceptive synergy (measured as a greater % reduction in flinches), whereby excess serotonin reduced this effect (Shen et al., 2013). The complexity of tramadol pharmacodynamics, even before the complexity conferred by pharmacokinetics, suggests that its possible tramadol-induced serotonin and norepinephrine reuptake inhibition may have been the primary pathway of antinociception in this paradigm of oral dosing, while under other dosing regimes (for example i.p.) the μ -opioid receptor activity of ODSMT may predominate.

We first tested tramadol analgesia at 40 mg/kg i.p. based on previously published data, then increased the dose to 100 mg/kg (2.5-fold) to match peak analgesia (Figure 1 and Wang et al. 2015). However, under these conditions i.c.v. propranolol 24 hr pretreatment altered serum drug and metabolite levels (Figure 2), suggesting an interaction between centrally administered propranolol 24 hr pretreatment and high dose oral tramadol. This propranolol 24 hr pretreatment before oral tramadol appeared to affect the absolute bioavailability of oral tramadol not via hepatic metabolism. Long term exposure to propranolol in healthy adults (40 mg 4 times daily for 1 week) can increase gastric emptying (decreased emptying $t_{1/2}$) (Rees et al., 1980) so its possible that some i.c.v. propranolol given prior to oral tramadol had an impact on gastric motility leading to higher serum, and resulting CNS, levels of

tramadol and its metabolites. However, propranolol is given 24 hr prior to tramadol dosing, has a short $t_{1/2}$ of 1 hour in rat brain and plasma, and has been previously shown, using this paradigm, to have no impact on peripheral drug metabolism or drug levels (Bianchetti et al., 1980, Zhou et al., 2013, McMillan and Tyndale, 2015, Miksys et al., 2017, McMillan et al., 2019). This suggests alternative, as yet unidentified, drug interactions resulted with oral tramadol.

While this work examined the impact of brain CYP2D metabolism on acute oral tramadol effect as a proof of concept study, future extensions into additional pain models and chronic administration paradigms could further demonstrate the importance of brain tramadol metabolism in altering drug response. Our study used the tail-flick reflex assay as a measure for tramadol antinociception in vivo. While the tail-flick test is spinally mediated, we have previously used the hot plate test as a measure of supra-spinally mediated opioid-induced antinociception and demonstrated an impact of brain CYP2D metabolism in this model as well; inducing brain CYP2D with seven-day subcutaneous nicotine administration increased codeine-induced antinociception by both tail-flick reflex (1.6-fold greater $AUC_{0-30 \text{ min}}$) and hot-plate test (2.5-fold greater AUC) (McMillan and Tyndale, 2015, Deuis et al., 2017). However, there may be merit in extending this model of brain CYP2D-mediated tramadol drug effect to tests with greater face validity to clinical pain, i.e. non-reflexive pain tests like the formalin paw-injection, or neuropathic pain using chronic constrictive injury of the sciatic nerve (Deuis et al., 2017). Of note, mice given i.p. tramadol exhibited analgesia in the formalin paw-injection model of acute pain; the analgesic effect of tramadol was reversed by serotonin receptor (5-HT₂R) antagonist ketanserin but not naloxone (non-specific opioid receptor antagonist) (Oliva et al., 2002). This suggests that altering brain CYP2D metabolism of tramadol may, similar to effects seen in tail-flick, alter pain response by way of serotonin/norepinephrine reuptake independent of opioid receptor activity of O-DSMT. Chronic tramadol use is associated with adverse effects like loss of drug potency (analgesic tolerance), as well as respiratory depression and seizures. We have previously shown that brain CYP2D metabolism alters chronic opioid-induced tolerance, proportional to its impact on acute opioid-induced analgesic response (McMillan and Tyndale, 2017). As tramadol, like many analgesics, is often prescribed for use in long term pain management, the role of brain CYP2D on chronic tramadol-induced response and adverse effects remains an important consideration in the scope of tramadol use clinically (McCarberg, 2007).

This study provides further evidence of an impact for rat brain CYP2D on centrally acting drug metabolism and response. Together, with previous findings that i.p. tramadol-induced analgesia may be due primarily to the CYP2D metabolite ODSMT, our data adds a level of complexity to pharmacokinetic/pharmacodynamic considerations in extrahepatic drug metabolism and response. Due to the large number of centrally acting compounds that are metabolized by, and inhibitors of, CYP2D6 in humans and having shown that brain CYP2D activity impacts oral tramadol analgesia, it is possible that drug-drug interactions, including those within the brain, may impact tramadol response in a clinically significant manner. While in vitro and in vivo drug interaction studies show a sufficient impact of CYP2D6 inhibitors (e.g. fluoxetine and paroxetine) on peripheral tramadol metabolism, the full pharmacological impact is unknown (Laugesen et al., 2005, Dean, 2012). In this paradigm using the pharmacologically unique opioid tramadol, enantiomer-specific metabolism, an

additive/synergistic antinociceptive effect of multiple receptor systems, and transporter drug-drug interactions may all play some role in the impact that CYP2D-mediated metabolism within the brain plays in tramadol response.

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Abbreviations:

CYP	Cytochrome P450
NDSMT	N-desmethyltramadol
ODSMT	O-desmethyltramadol

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Highlights

- Tramadol is an opioid with inhibitory actions on serotonin/norepinephrine reuptake
- Role of CYP2D metabolism on tramadol analgesia unclear; brain CYP2D may contribute
- Inhibiting rat brain-specific CYP2D with propafenone increased tramadol analgesia
- Increased analgesia consistent with higher brain tramadol levels by microdialysis
- Parent tramadol appears to be responsible for analgesia in oral administration

Figure 1: Oral tramadol dose response.

a) Oral tramadol dose response curves for 60 to 120 mg/kg p.o. b) Peak analgesia for each oral tramadol dose compared to tramadol at 40 mg/kg i.p.; the tramadol i.p. dose of 40 mg/kg was selected based on previous publication by Wang et al. (2015). Using this dose response data, we selected the tramadol p.o. dose of 100 mg/kg to test in subsequent experiments, which enabled assessment of an increase or decrease of response following inhibition of CYP2D in the brain. %MPE, percentage of maximal possible effect; SD, standard deviation.

Figure 2: Pretreatment effects on serum drug levels.

Propranolol, but not propafenone, pretreatment appeared to increase serum tramadol and metabolite levels. Compared with vehicle pretreatment (V), propranolol pretreatment (PRL) resulted in an apparent increase in a) the parent tramadol (TRAM, ANOVA pretreatment (PT) $p=0.18$; a), and metabolites b) O-desmethyltramadol (ODSMT, PT $p=0.17$) and c) N-desmethyltramadol (NDSMT, PT $p=0.25$) levels for the 30 min after tramadol gavage (100 mg/kg p.o., $n=5$ /group). Compared with V pretreatment, propafenone pretreatment (PRF) did not alter serum d) TRAM (PT $p=0.64$), e) ODSMT (PT $p=0.54$), or f) NDSMT (PT $p=0.91$) levels after tramadol gavage ($n=4$ /group). SD, standard deviation.

Figure 3: Acute competitive inhibition of CYP2D in brain increased oral tramadol-induced analgesia.

a) Compared with vehicle pretreatment (V), propafenone pretreatment (PRF) resulted in a significant increase in analgesia (ANOVA pretreatment (PT) from 0–60 min $p=0.02$) after tramadol gavage (100 mg/kg p.o., $n=11$ within-animal). b) PRF pretreatment resulted in a significant and consistent ($N=10/11$) increase in analgesia AUC from 0–60 min after oral tramadol, as indicated by individual-animal analgesia AUC data. %MPE, percentage of maximal possible effect; SEM, standard error of the mean.

Figure 4: Propafenone pretreatment increased brain tramadol levels and associated tramadol-induced analgesia.

a) Compared with vehicle pretreatment (V), propafenone pretreatment (PRF) resulted in an apparent increase in free brain tramadol levels (TRAM, ANOVA pretreatment (PT) $p = 0.27$) after tramadol gavage. b) PRF pretreatment did not significantly alter free brain O-desmethyltramadol (ODSMT, PT $p = 0.62$) or c) N-desmethyltramadol (NDSMT, PT $p = 0.60$) levels after tramadol gavage. d) In agreement with Figure 3, where the experiments were powered to examine analgesia, PRF pretreatment trended towards ($N=5/6$) an increase in tramadol-induced analgesia at 30 min (peak) after tramadol gavage (PT $p = 0.09$; d). MD values were adjusted for in vitro and in vivo probe recovery. %MPE, percentage of maximal possible effect; SEM, standard error of the mean.