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# Human CYP2D6 in the Brain Is Protective Against Harmine-Induced Neurotoxicity: Evidence from Humanized CYP2D6 Transgenic Mice

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## Abstract

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 with wild-type mice (WT). We first sought to elucidate the role of CYP2D in general within the brain in harmine-induced hypothermia and tremor severity. A 4-h intracerebroventricular (ICV) pretreatment with the CYP2D inhibitor propranolol increased harmine-induced hypothermia and tremor in TG and increased harmine-induced hypothermia in WT. We next sought to specifically demonstrate that human CYP2D6 expressed in TG brain

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Compliance with Ethical Standards

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altered harmine response severity. A 24-h ICV propranolol pretreatment, which selectively and irreversibly inhibits human CYP2D6 in TG brain, increased harmine-induced hypothermia. This 24-h pretreatment had no impact on harmine response in WT, as propranolol is not an irreversible inhibitor of mouse CYP2D in the brain, thus confirming no off-target effects of ICV propranolol pretreatment. Human CYP2D6 activity 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

## Introduction

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-phe-nyl-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 with 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-h 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-h 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 h 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.

## Methods

### Animals

TG were produced by microinjecting fertilized FVB/N mouse eggs with an insert that included one copy of 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-h 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.

### Intracerebroventricular 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.

### 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/min 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 min before injectors were removed and cannula dummies replaced. In rodents, ICV injected compounds (e.g., dyes and CYP inhibitors) diffuse bilaterally and produce CYP inhibition across brain regions (e.g., cerebellum to frontal cortex) [16, 37, 38]. ICV pretreatment injections were given either 24 or 4 h 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, because this dose allowed for the detection of an increase or decrease in harmine response.

### Harmine-Induced Hypothermia and Tremor Assessment

Hypothermia was calculated as the change in body temperature compared with a baseline, where baseline was measured 15 min 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 s 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).

### 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 h 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-min intervals for 90 min.

### 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 with the effect size observed following 4-h pretreatment. TG 0n 25) and WT (n = 24) mice received ICV propranolol (or cyclodextrin vehicle) pretreatment 24 h 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-min intervals for 90 min.

### Four- and 24-h ICV Pretreatment and In Vitro Brain and Liver CYP2D Activity

Four- or 24-h ICV propranolol (or cyclodextrin vehicle) pretreatment was administered to TG and WT ($n$ =5–6 per pretreatment per mouse line for 4- and 24-h experiments). Mice were then euthanized, and the cerebellums and livers were collected. The cerebellum was used to assess CYP2D activity in vitro in brain, due to high CYP2D expression and activity in this region in rodents [29, 39]. 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, 40, 41]. 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 [42], with $K_m$ values of 4.5 and 3.6 μM in TG and WT, respectively [35], similar to the ~ 5 μM $K_m$ in human liver microsomes [43]. 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 (ACSF) (pH 7.4) for 90 min 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 min 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 (72.8% dextrorphan recovery), 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].

### Statistical Analysis

All analyses were performed using Prism6 (GraphPad version 6.0c, La Jolla, CA, 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 × 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 × pretreatment). Repeated-measure (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 ($AUC_{0–90}$) was analyzed across all groups (mouse line × pretreatment) using two-factor mixed-design ANOVAs. $AUC_{0–90}$ 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 $AUC_{0–90}$ bytherecordedresponsedura-tion (90 min). 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) usingunpairedtwo-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.

## Results

### 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.

### 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 $AUC_{0–90}$ ($F(1, 22) = 67.6$, $p < 0.0001$), due to

propranolol (versus vehicle) pretreatment increasing $AUC_{0–90}$ in TG($t$ (15) = 9.42, $p$ <0.0001)andinWT($t$(7) = 3.46, $p$ = 0.0212) (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) = 3.34, $p$ = 0.025) corresponded to a 1.3-fold increase in apparent harmine dose (Fig. 3d). There was no effect of ICV propranolol (versus vehicle) pretreatment on baseline body temperature measured before IP harmine (or distilled water) treatment ($p$ > 0.05; data not shown).

Hypothermia was more severe in vehicle-pretreated WT compared with TG (mouse line, $F$(1, 22) = 6.69, $p$ =0.0168) Fig. 3a), and the $AUC_{0–90}$ was greater in vehicle pretreatment WT compared with TG ($t$(22) = 2.49, $p$ = 0.0209) (Fig. 3b). 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) (Online Resource 1).

## 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. 4a). There was an interaction effect of pretreatment and mouse line on hypothermia, evaluated by $AUC_{0–90}$ ($F$(1, 47) = 12.2, $p$ = 0.001), as well as a main effect of pretreatment ($F$(1, 47) = 19.1, $p$ < 0.0001), due to propranolol (versus vehicle) increasing AUC in TG ($t$(24) = 7.06,$p$ < 0.0001)(Fig. 4b). 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. 4c). In propranolol (versus vehicle) pretreated WT, there was no change in mean response (Fig. 4d). There was no effect of ICV propranolol (versus vehicle) pretreatment on baseline body temperature measured before IP harmine treatment ($p$ > 0.05; data not shown).

Hypothermia was more severe in vehicle-pretreated WT compared with TG (mouse line, $F$(1, 47) = 8.09, $p$ =0.0066) (Fig. 4a). The AUC was greater in vehicle-pretreated WT compared with TG ($t$(38.47) = 2.73, $p$ = 0.0095) (Fig. 4b).

## 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. 5a). There was a main effect of pretreatment on tremor, evaluated by AUC ($F$(1, 22) = 9.61; $p$ = 0.0052), due to propranolol (versus vehicle) pretreatment increasing $AUC_{0–90}$ in TG($t$(15) = 4.96,$p$= 0.0004) (Fig. 5b). 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. 5c). In propranolol (versus vehicle) pretreated WT, there was no change in mean response (Fig. 5d). Propranolol (versus vehicle) pretreatment also increased peak tremor (measured at 15 min

after harmine injection) in TG ($t(15) = 4.20$, $p = 0.0008$) (Online Resource 2a), but not in WT (Online Resource 2b).

Tremor was more severe in vehicle-pretreated WT compared with TG (mouse line, $F(1, 22) = 20.4$, $p = 0.0002$) (Fig. 5a). There was a main effect of mouse line on tremor, evaluated by $AUC_{0–90}$ ($F(1,22)=13.6$,$p=0.0013$),due to $AUC_{0–90}$ being greater in vehicle-pretreated WT compared with TG ($t(22) = 4.38$, $p = 0.0002$) (Fig. 5b).

### 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. 6a). Propranolol (versus vehicle) pretreatment had no effect on tremor, evaluated by $AUC_{0–90}$ (Fig. 6b) or on mean response of either TG or WT (Fig. 6c, d). Propranolol (versus vehicle) pretreatment trended towards increasing peak tremor in TG ($t(24) = 1.77$, $p = 0.0887$) (Online Resource 2c), but not in WT (Online Resource 2d).

Tremor was more severe in vehicle-pretreated WT compared with TG (mouse line, $F(1, 47) = 7.48$, $p = 0.0088$) (Fig. 6a). There was a main effect of mouse line on tremor, measured by $AUC_{0–90}$ ($F(1, 47) = 15.5$, $p = 0.0003$), due to $AUC_{0–90}$ being greater in vehicle-pretreated WT compared with TG ($t(47) = 2.73$, $p = 0.0089$) (Fig. 6b).

### Effect of ICV Pretreatment on In Vitro CYP2D Enzymatic Activity in the Brain and in the 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. 7a). There was no effect of 4-h ICV propranolol (versus vehicle) pretreatment in WT (Fig. 7b). Twenty-four-hour ICV propranolol (versus vehicle) pretreatment reduced in vitro brain dextrorphan formation in TG ($t(10) = 2.63$, $p = 0.0253$) (Fig. 7c), but not in WT (Fig. 7d). Taken together, the effects of the 4-h and 24-h 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 4- or 24-h ICV propranolol (versus vehicle) pretreatment on in vitro liver dextrorphan formation in TG or WT (Fig. 7e–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.

## Discussion

To study the role of CYP2D in general within the brain in harmine-induced hypothermia and tremor responses, a 4-h pretreatment with ICV propranolol was given. This 4-h 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-h pretreatment with ICV propranolol was given. This 24-h 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 and are detected at picogram to nanogram concentrations in various human tissues [22, 44–46]. Beta-carbolines can be neurotoxic in vitro and in vivo [47, 48]. Many beta-carbolines are structurally similar to MPTP and can be bioactivated in the brain to *N*-methyl-beta-carbolinium cations [46], which are analogs of the Parkinsonism-inducing neurotoxic metabolite $MPP^+$ [10, 49]. 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, 50]. Harmine is a beta-carboline and a metabolite of harmane[51], and both are present in human tissue [45]. Harmane levels are higher in those with essential tremor [23]and Parkinson’s disease [52]. 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, 53]. Many neurotoxins, including MPTP and chlorpyrifos, induce hypothermia via central mechanisms [54, 55]. A 4-h 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-h 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. Although harmine levels were not measured in brain, the findings suggest 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 with 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-h 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-h ICV propranolol pretreatment. The impact of 24-h 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 [56]. Beta-carbolines have been causally implicated in essential tremor etiology [57], as levels in blood [58] and in the brain [23] were elevated in those with essential tremor compared with healthy controls. Furthermore, among a group of individuals with essential tremor, beta-carboline levels in blood were associated with lower metabolic function, indicating more degeneration, in the cerebellum [21]. In fact, cerebellar degeneration is a common feature of essential tremor [59] and of beta-carboline administration in animal models [60]. Beta-carbolines, including harmine and its congener harmaline, are commonly used in rodents to model essential tremor [57, 61].

The 4-h 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-h 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-h pretreatment in WT, especially if the effect was not robust.

Following a 24-h 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-h pretreatment compared with the 4-h 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-h 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. 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.

Propranolol is used as a treatment for essential tremor in humans and, given as a 30-min pretreatment, it suppresses harmine-induced tremor in rodents [62]. After a 24-h ICV pretreatment, propranolol does not remain in the brain, due to its ~ 2-h half-life in mouse brain [63], and should have no direct suppressant effect on harmine-induced tremor. Given as a 4-h pretreatment, propranolol remains in the brain and, if in sufficient amounts, would have suppressed tremor. The 4-h ICV propranolol pretreatment enhanced tremor in TG, suggesting that the tremor-suppressant effect was diminished after 4 h, or that the enhancement of tremor superseded this effect. Propranolol pretreatments could also have indirectly altered harmine responses via inhibition of CYP2D6- and/or CYP2D-mediated metabolism of endogenous compounds. For example, CYP2D isozymes may contribute to alternative pathways of serotonin and/or dopamine synthesis in the brain, which has been tested under specific conditions [64, 65]. Reduced serotonin and dopamine signaling would attenuate harmine-induced hypothermia and tremor [24, 66]; thus, inhibiting CYP2D6 and/or CYP2D in TG and WT brain, if sufficient to meaningfully reduce serotonin and dopamine synthesis, should have reduced harmine-induced hypothermia and tremor severity. However, inhibitor pretreatment exacerbated these responses, consistent with propranolol inhibiting harmine metabolism and increasing harmine in the brain. Although brain drug levels were not measured, we used several controls that, taken together, suggest there were no relevant off-target effects of propranolol on body temperature in general, or on harmine-induced hypothermia and tremor.

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 [67, 68]. 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, 69]. 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 [70], 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 [59] and Parkinson's disease [71]. Cigarette smoking is associated with decreased risk for

essential tremor [72]and Parkinson's disease [73], 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 [74], as well as the formation of dopamine from tyramine [75] and serotonin from 5-methoxytryptamine, with evidence for the latter occurring in vivo [65, 76]. 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, 77]. 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 [78]. Genetic *CYP2D6* extensive and ultra-rapid metabolizers were also more frequent among patients with eating disorders compared with healthy controls [79]. 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.

Using this novel approach, we demonstrated previously 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 also 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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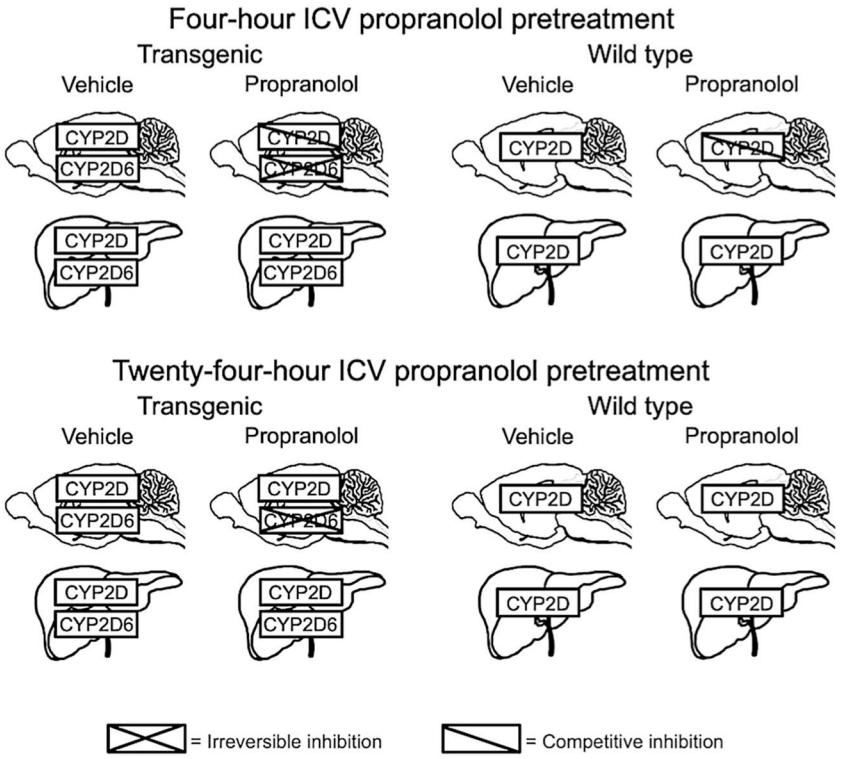


**Fig. 1.**

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 4-and 24-h ICV propranolol pretreatments, there is no inhibition of human CYP2D6 in TG liver, or of mouse CYP2D in TG or WT liver, indicating that propranolol given ICV does not reach the liver in sufficient amounts to alter hepatic metabolism. For more details, see text and Tolledo et al. [34]

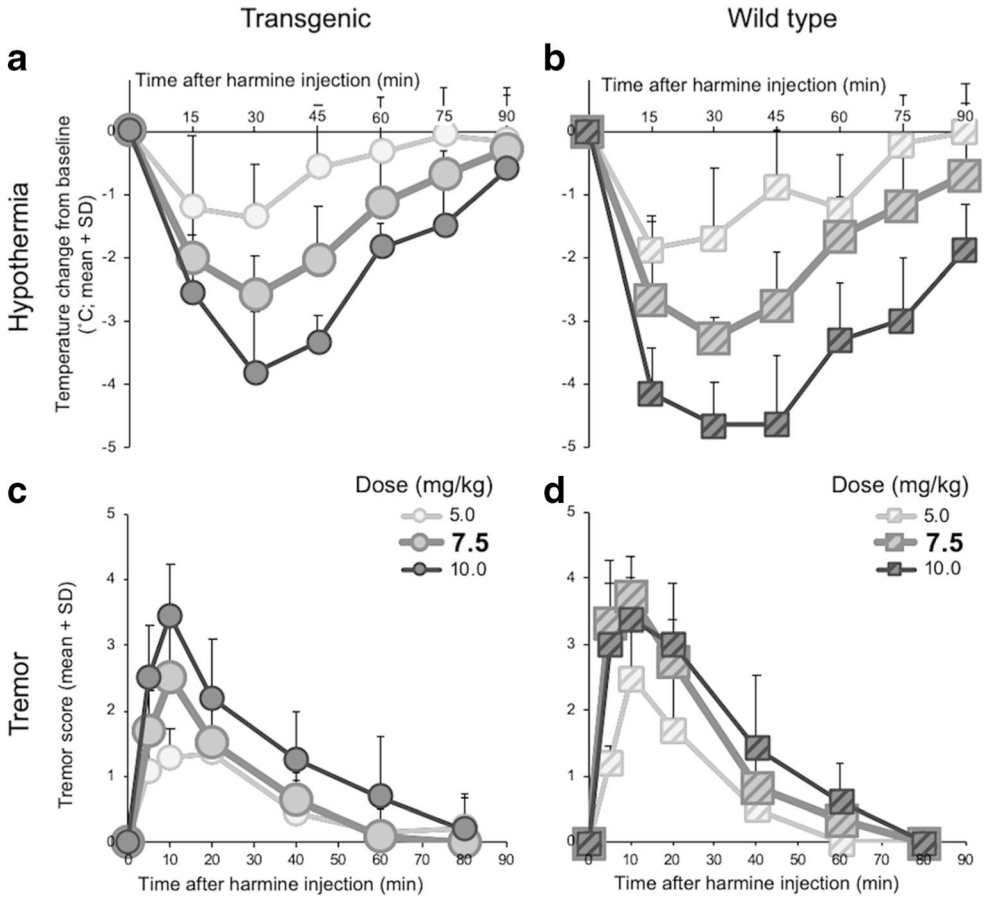


**Fig. 2.**

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 c TG and d 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

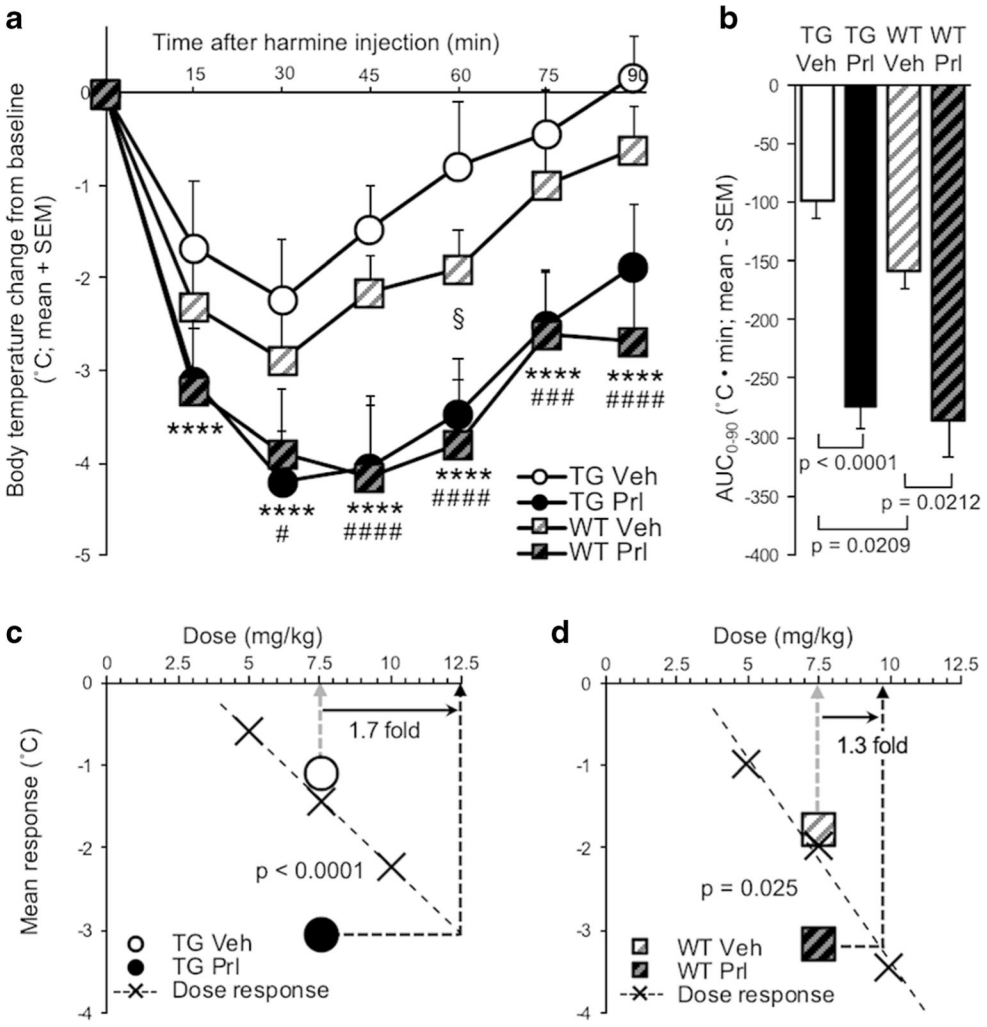


**Fig. 3.**

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 min in TG and WT. **b** $AUC_{0–90}$ 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. 2a, b, 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: §$p < 0.05$. Vehvehicle, Prl propranolol, SEM standard error of the mean, AUC area under the curve

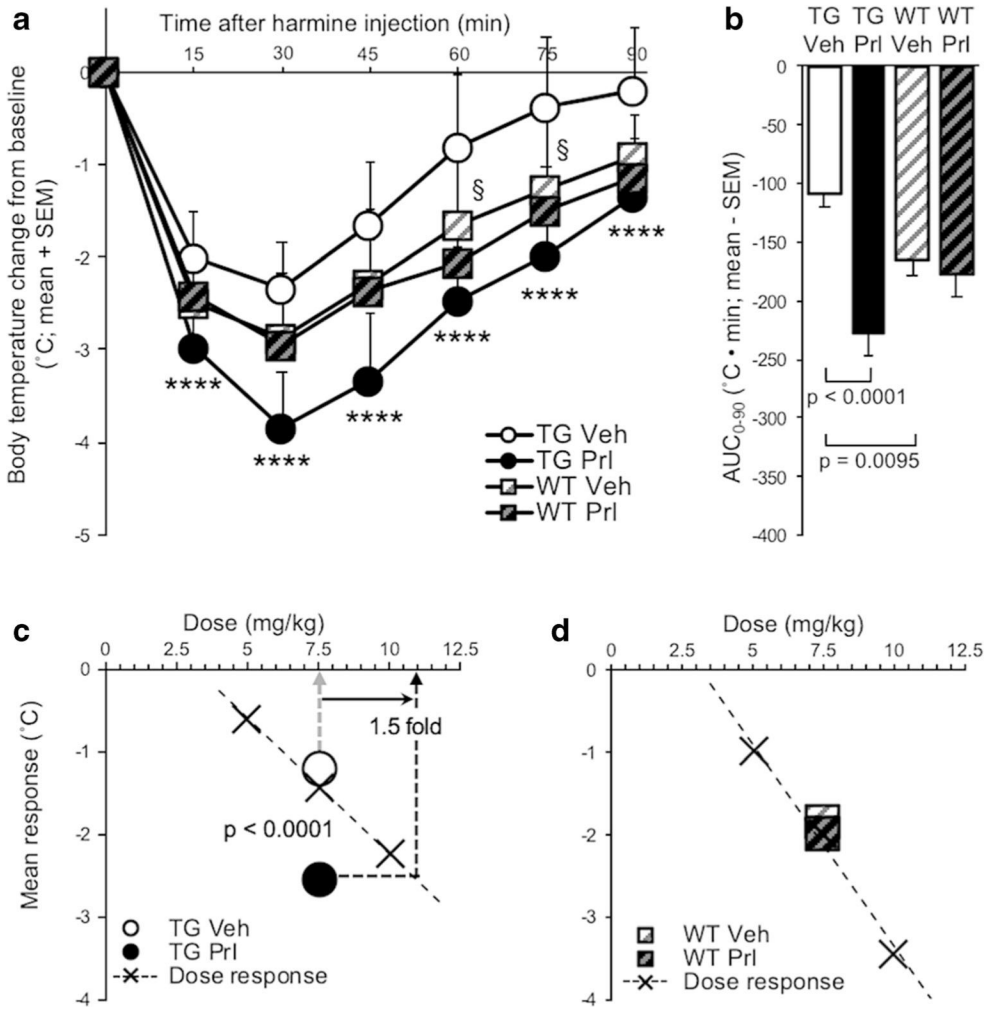


**Fig. 4.**

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 min in TG and WT. **b** $AUC_{0–90}$ 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. 2a, b, respectively. Propranolol versus vehicle-pretreated TG: ***$p < 0.001$, ****$p < 0.0001$; vehicle-pretreated WT versus TG: §$p < 0.05$. Vehvehicle, Prl propranolol, SEM standard error of the mean, AUC area under the curve

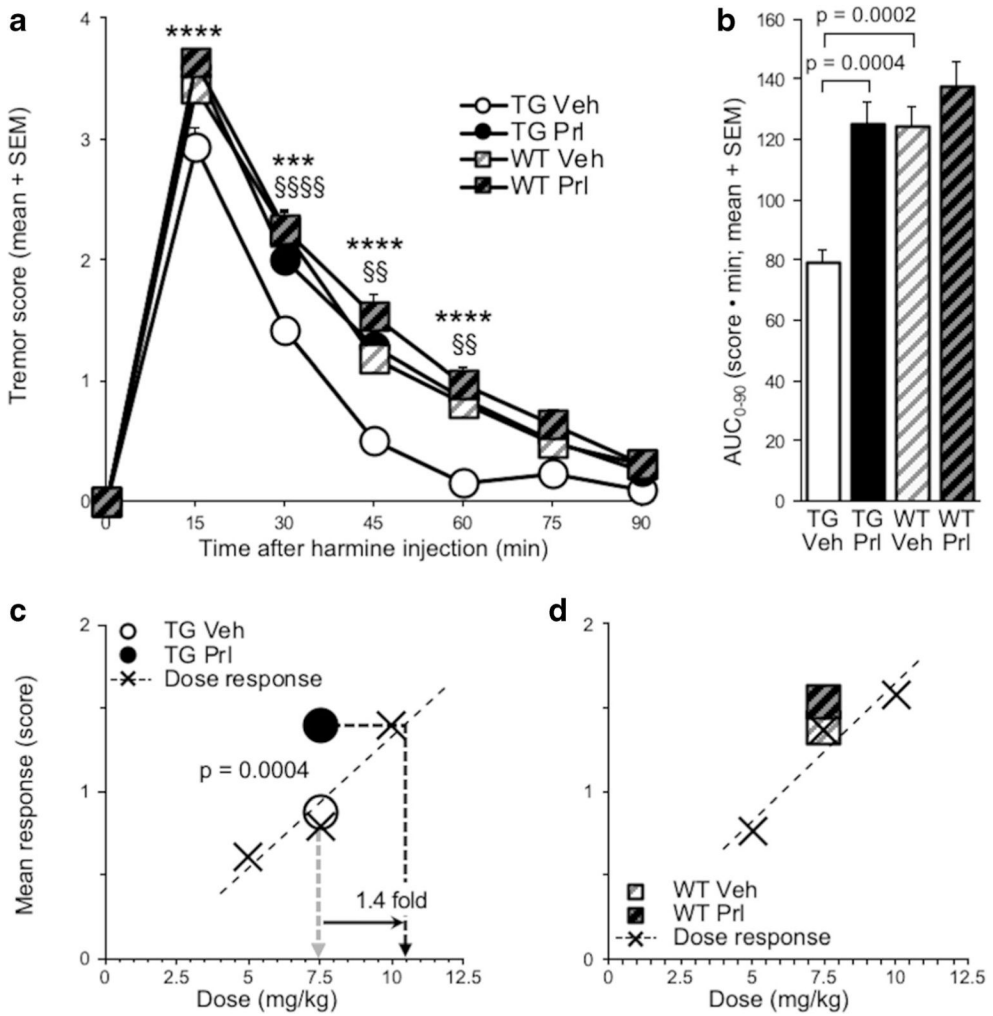


**Fig. 5.**

Four-hour ICV propranolol (versus vehicle) pretreatment exacerbated harmine-induced tremor in TG. **a** Four hours after ICV propranolol or vehicle pretreatment, harmine treatment was administered, and tremor was scored for 90 min in TG and WT. **b** $AUC_{0–90}$ 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. 2c, d, 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

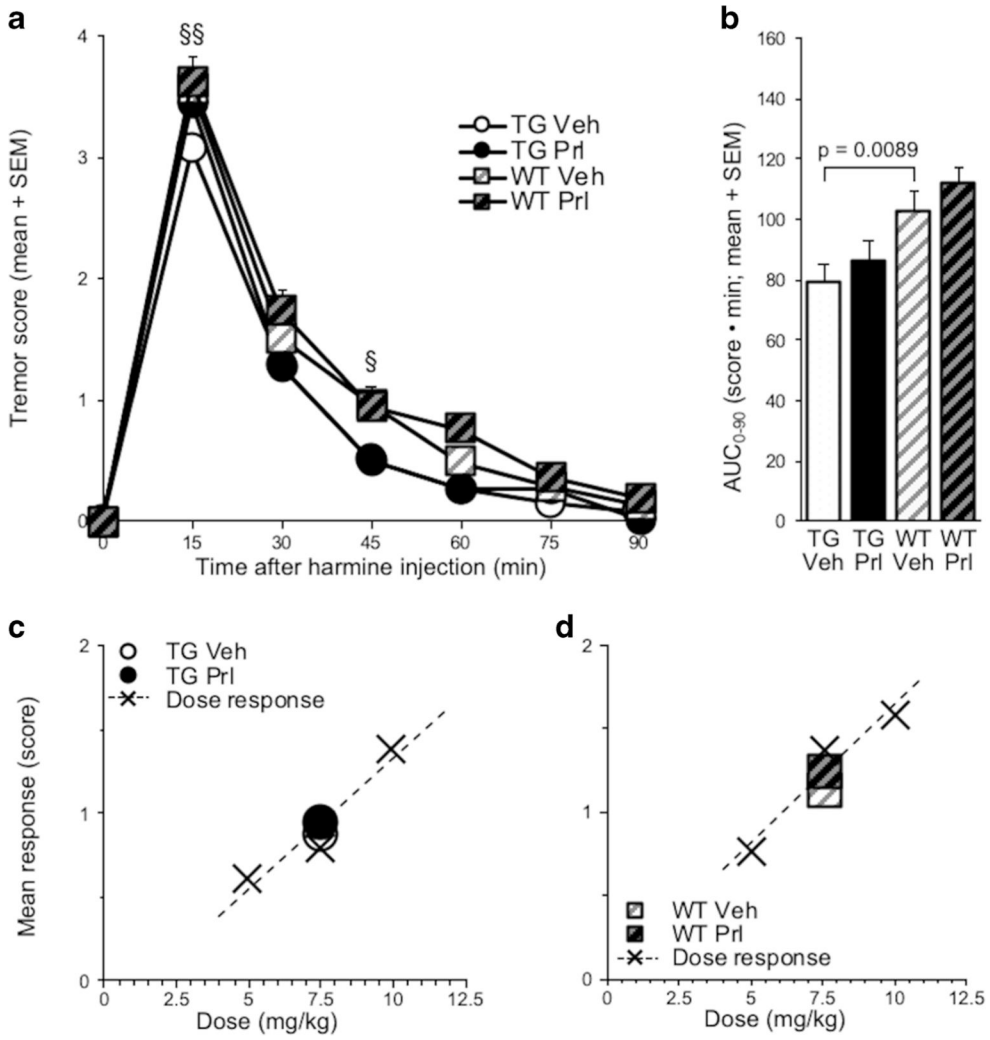


**Fig. 6.**

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 min in TG and WT. **b** $AUC_{0–90}$ 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, d, 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

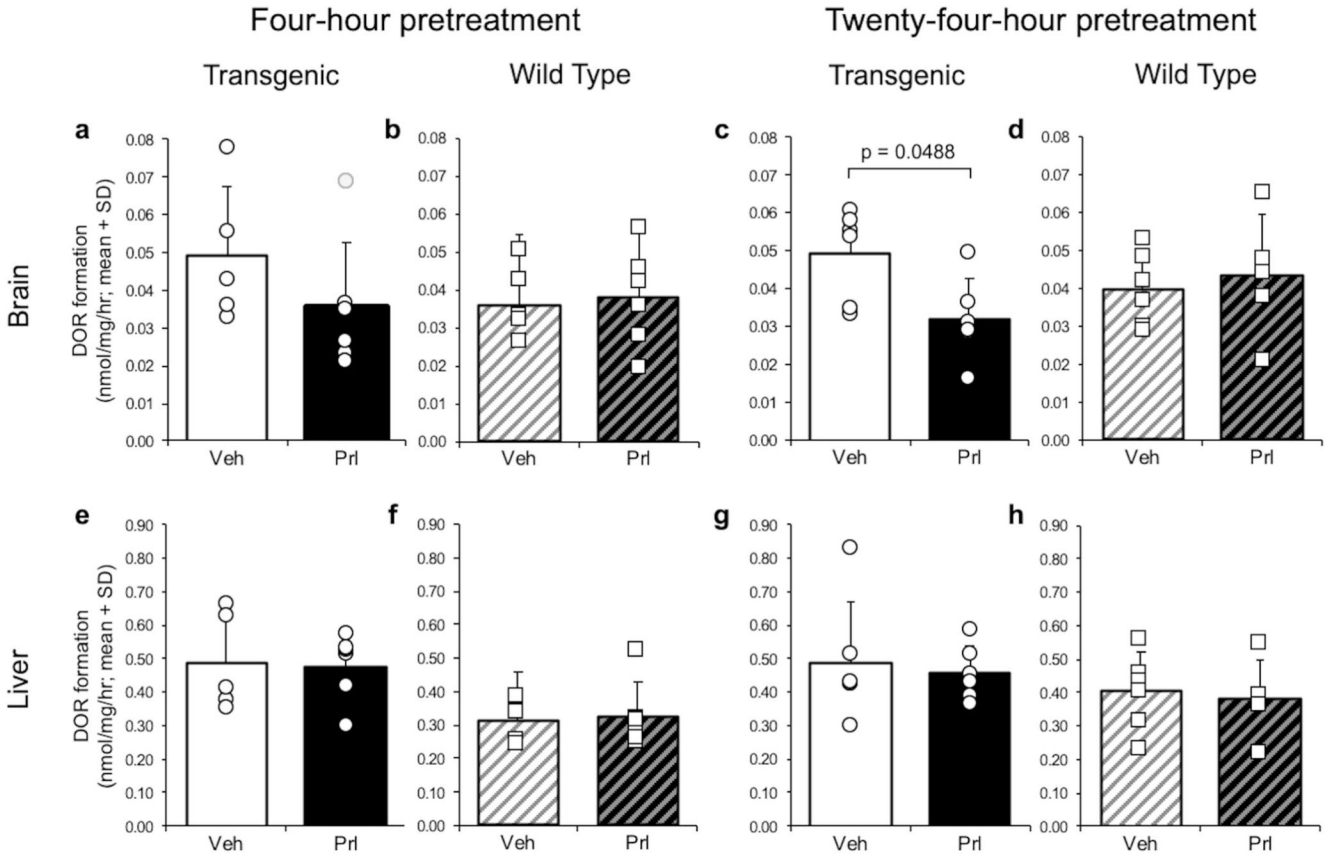


**Fig. 7.**

ICV propranolol (versus vehicle) pretreatment reduced in vitro brain CYP2D activity in TG. In vitro brain dextrorphan formation after 4-h ICV propranolol or vehicle pretreatment in **a** TG (outlier in gray) and **b** WT, and after 24-h ICV pretreatment in **c** TG and **d** WT. In vitro liver dextrorphan formation after 4-h ICV propranolol or vehicle pretreatment in **e** TG and **f** WT, and after 24-h ICV pretreatment in **g** TG and **h** WT. Data were normalized to the vehicle-pretreated TG group in the 4-h ICV pretreatment experiment. DOR dextrorphan, Veh vehicle, Prl propranolol, SD standard deviation