

Short Communication

Opioid Analgesia in P450 Gene Cluster Knockout Mice:

A Search for Analgesia-Relevant Isoforms

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Abbreviations: AUC, area under the curve; BCPRN, brain cytochrome P450 reductase null;

³HCIM, ³H-cimetidine; KO, knockout; icv, intracerebroventricular; WT, wild-type.

ABSTRACT

Cytochrome P450 monooxygenases (P450s), which are well known drug-metabolizing enzymes, are thought to play a signal transduction role in μ opioid analgesia and may serve as high-affinity ^3H -cimetidine ($^3\text{HCIM}$) binding sites in the brain. $^3\text{HCIM}$ binding sites may also be related to opioid or non-opioid analgesia. However, of the more than 100 murine P450 enzymes, the specific isoform(s) responsible for either function have not been identified. Presently, 3 lines of constitutive P450 gene cluster knockout (KO) mice with full-length deletions of 14 *Cyp2c*, 9 *Cyp2d* and 7 *Cyp3a* genes were studied for deficiencies in $^3\text{HCIM}$ binding and for opioid analgesia. Liver and brain homogenates from all three genotypes showed normal $^3\text{HCIM}$ binding values, indicating that gene products of *Cyp2d*, *Cyp3a* and *Cyp2c* are not $^3\text{HCIM}$ - binding proteins. *Cyp2d* KO and *Cyp3a* KO mice showed normal antinociceptive responses to a moderate systemic dose of morphine (20 mg/kg, s.c.), thereby excluding 16 P450 isoforms as mediators of opioid analgesia. In contrast, *Cyp2c* KO mice showed a 41% reduction in analgesic responses following systemically (s.c.) -administered morphine. The significance of brain *Cyp2c* gene products in opioid analgesia is uncertain, however, since little or no analgesic deficits were noted in *Cyp2c* KO mice following intracerebroventricular (icv) or intrathecal morphine administration, respectively. These results show that gene products of *Cyp2d* and *Cyp3a* do not contribute to μ opioid analgesia in the CNS. A possible role for *Cyp2c* gene products in opioid analgesia requires further consideration.

Introduction

Opioid analgesics such as morphine act on μ opioid receptors in the brain and spinal dorsal horn to relieve pain. In the periaqueductal grey, μ receptor stimulation reduces pre-synaptic GABAergic activity, leading to activation of descending, pain-dampening circuits (Heinricher and Ingram, 2008), but the relevant cellular mechanisms for this effect remain uncertain. We recently described the development of brain cytochrome P450 reductase null (BCPRN) mice (which lack brain neuronal P450 activity) and showed that these mice exhibit defective analgesic responses to morphine (Conroy, et al., 2010). P450s are best known as mediators of drug metabolism, but morphine is not metabolized by P450s (Kuo, et al., 1991), and brain levels of morphine were identical in BCPRN and control mice (Conroy, et al., 2010). Since P450s also catalyze the oxidation of endogenous lipids (Spector, 2009), a pharmacodynamic (vs. pharmacokinetic) mechanism best explains the brain P450 requirement for opioid analgesia. Conroy et al. (2010) proposed that μ opioids act in the CNS by stimulating the release and P450-mediated epoxidation of arachidonic acid. The epoxide products may have pain-relieving properties (Terashvili, et al., 2008; Wagner, et al., 2013). Subsequent results support this P450/epoxygenase hypothesis for μ agonist action (Zhang and Pan, 2012; Conroy, et al., 2013). However, it is not known which of the more than 100 P450 isoforms in the mouse (Nelson, et al., 2004) might be important for opioid analgesia.

Earlier work from one of our labs focused on the analgesic properties of the histamine H₂ receptor antagonist cimetidine and its congener impropgan (Hough, 2004). ³H-Cimetidine (³HCIM) exhibits high affinity, specific binding to unknown protein(s) in brain and liver which are distinct from the H₂ receptor. Characterization of ³HCIM -binding proteins in brain suggested a P450 profile, but these were never identified (Stadel, et al., 2008). Identification of

these proteins is of interest since they could be new analgesic drug targets (Hough, et al., 2007). Although this idea has not been confirmed (Stadel, et al., 2010), ³HCIM –binding studies led to the discovery that the pain-relieving effects of both non-opioid (Hough, et al., 2011) and opioid (Conroy, et al., 2010) analgesic drugs require brain P450 activity.

The abundance of murine P450 genes makes it difficult to assign specific roles to particular isoforms, but the recent development of several P450 gene cluster KO mice is important progress toward solving this problem. Constitutive KO genotypes were recently described for the *Cyp2c*, *Cyp2d* and *Cyp3a* gene clusters (Scheer, et al., 2012a, 2012b; Hasegawa, et al., 2011; van Herwaarden, et al., 2007). Experiments with these new models (*Cyp2c* KO, *Cyp2d* KO, and *Cyp3a* KO mice) are clarifying the roles for enzyme families in the metabolism of xenobiotics. Presently, we used these three lines of KO mice to study two P450-related problems: 1) assess possible mechanistic roles for CYP2C, CYP2D, and/or CYP3A subfamilies in the pain-relieving actions of morphine, and 2) search for ³HCIM -binding proteins in liver and brain homogenates from these mouse lines.

Materials and Methods

Drugs and solutions. Morphine sulfate (Mallinckrodt, St. Louis, MO) was dissolved in saline.

Animals. Three recently-developed lines of constitutive P450 gene cluster KO mice (*Cyp2c*, *Cyp2d*, and *Cyp3a*) along with controls (C57BL/6Ntac, Taconic B6-M) were purchased from Taconic Biosciences (Germantown, NY). *Cyp2c* KOs (B6-*Cyp2c*^{tm1104Arte}, Taconic 9177-M) contain homozygous deletions of 14 full-length P450 genes (*Cyp2c55*, *2c65*, *2c66*, *2c29*, *2c38*, *2c39*, *2c67*, *2c68*, *2c40*, *2c69*, *2c37*, *2c54*, *2c50*, *2c70*), but retain a functional *Cyp2c44* (Scheer, et al., 2012a). *Cyp2d* KOs (C57BL/6-Del(15*Cyp2d*22-*Cyp2d*26)1Arte, Taconic 9178-M)

contain homozygous deletions of 9 full-length P450 genes (*Cyp2d22*, *Cyp2d11*, *Cyp2d10*, *Cyp2d9*, *Cyp2d12*, *Cyp2d34*, *Cyp2d13*, *Cyp2d40* and *Cyp2d26*) as recently described (Scheer, et al., 2012b). *Cyp3a* KOs (C57BL/6NTac-Del(5*Cyp3a57*-*Cyp3a59*)1Arte, Taconic 8841-M) contain homozygous deletions of 7 full-length P450 genes (*Cyp3a11*, *Cyp3a16*, *Cyp3a25*, *Cyp3a41*, *Cyp3a44*, *Cyp3a57*, *Cyp3a59* but have retained *Cyp3a13*) as described (Hasegawa, et al., 2011). All subjects were adult males (greater than 10 weeks of age, 19 – 44 g). Animals were maintained on a 12-h light/ dark cycle (lights on from 0700 to 1900), with food and water freely available. All animal experiments were approved by the Institutional Animal Care and Use Committee of Albany Medical College.

Surgery. Cannulas were chronically implanted for icv micro-injections. Following anesthesia (pentobarbital sodium, 50 mg/kg, i.p., supplemented with isoflurane), a stainless steel guide cannula was stereotaxically inserted into the left lateral ventricle (coordinates were AP - 0.5, ML -1.0, DV -2.0 mm from bregma following Paxinos and Franklin [2001]). The cannula was anchored to the skull with stainless steel screws and dental cement. After surgery, subjects were individually housed and allowed to recover for at least 5 days before testing.

Icv drug injections. Subjects were lightly secured in a laboratory pad, the cannula stylet removed, and the injection cannula inserted so as to extend 1 mm beyond below the guide. Injections (2 μ l) were made over a 1 min period. One min later, the injection cannula was clipped and sealed approximately 2 mm above the juncture with the guide cannula. Successful injections were verified by the movement of an air bubble in the tubing. Following testing, animals received pentobarbital sodium (100 mg/kg, i.p.) and India Ink (2 μ l). Brains were removed to verify ventricular distribution of the ink. Data from animals with incorrect placements or unsuccessful injections were excluded.

Nociceptive testing. The hot water tail immersion test was used to measure morphine analgesia (Sewell and Spencer, 1976). Subjects were restrained in a conical polypropylene tube, the tip of the tail immersed (2–3 cm) into a 55°C water bath, and latency to sudden tail movement or withdrawal from the water was recorded. Cutoff latencies were 8s. Subjects were baseline tested, received single s.c. or icv injections, and were re-tested as described in the figure legends. Subjects were only used for a single experiment.

Radioligand binding. These experiments were performed following Stadel et al. (2008). Tissues were homogenized in 10 vols of Tris-HCl buffer (100 mM, 0.5 mM EDTA, pH 7.4) and centrifuged (26,000g, 15 min). Pellets were resuspended in the same volume of buffer, re-homogenized, re-centrifuged, and stored at -80° until use. Resuspended pellets (0.3 to 0.4 mg protein/tube) were incubated in a total volume of 0.1 ml containing 50 nM ³HCIM (80 Ci/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO) in 100 mM Tris-HCl pH 7.4 for 60 min on ice. Burimamide (30 μM) was used to evaluate nonspecific binding. Samples were rapidly filtered through GF/B filters and rinsed three times with 1.5 ml of ice-cold buffer. Filters were placed in 5 ml of Ecoscint (National Diagnostics, Atlanta, GA) and counted in a scintillation counter. Protein content was determined using the bicinchoninic acid method (Pierce Chemical, Rockford, IL).

Data analysis. Nociceptive data are expressed as latencies (s, mean ± S.E.M.) and also as antinociceptive areas under the curve (AUCs). AUCs were calculated as the sum of post-injection difference scores for the intervals specified in each experiment. Each difference score was computed as a post-injection latency minus baseline latency. All data were analyzed by analysis of variance (ANOVA), followed by post-hoc testing (Statistica, StatSoft, Tulsa, OK).

Bonferroni post-hoc testing was performed as permitted by ANOVA results. Figures were produced with Prism 5.04 (Graphpad, San Diego, CA).

Results

Cyp2c KO mice show deficient analgesic responses to systemically-administered morphine. A large systemic dose of morphine (20 mg/kg, s.c.) produced robust analgesia in four mouse genotypes lasting up to 2 hr (Fig. 1A). ANOVA (between groups [factor #1]: genotype; within groups [factor #2, repeated measures]: time) found significant main effects of genotype ($F_{3,10} = 4.07$, $P < 0.05$) and time ($F_{7,21} = 65.9$, $P < 0.001$), with no significant interaction terms. Subsequent analysis showed that the genotype effects were due to the reduced latencies in the Cyp2c KO data. Deficient responses in the Cyp2c KO group were most evident 3 hr after morphine administration, confirmed by post-hoc testing (Fig. 1A). Baseline latencies (zero time scores, Fig. 1A) were not different across the four genotypes by one-factor ANOVA.

Because the Cyp2c KO deficit in Fig. 1A was most apparent at the later time points after 20 mg/kg morphine, a lower dose (10 mg/kg, s.c.) was studied in wild-type control (WT) and Cyp2c KO mice (Fig. 1B). Mean latencies in the KO groups after both doses of morphine were smaller than WT responses at all times (Fig. 1B). ANOVA (between groups [factor #1]: genotype; [factor #2]: dose; within groups [factor #3, repeated measures]: time) found significant main effects of genotype ($F_{1,19} = 4.6$, $P < 0.05$), dose ($F_{1,19} = 12.1$, $P < 0.01$), and time ($F_{4,76} = 69.0$, $P < 0.001$), with a significant time by dose ($F_{4,76} = 8.0$, $P < 0.001$) interaction term. Genotype differences at specific times in Fig. 1B were not large enough to be statistically significant by post-hoc testing, but the significant genotype term in the ANOVA confirms the diminished morphine responses in the Cyp2c KO mice. AUCs (brackets in 1B) showed 40% and 14% reductions in mean Cyp2c KO responses to morphine at 10 and 20 mg/kg, respectively, as

compared with WT. However, the genotype-related terms in the ANOVA of AUCs did not reach statistical significance ($P=0.0625$ for main effect of genotype).

Deficient morphine analgesia in Cyp2c KO mice was not clearly present following icv administration. In order to confirm the genotype effects seen after s.c. administration, morphine was administered directly into the CNS. Morphine (2 and 10 μg , icv) produced prolonged, dose-dependent analgesia in WT mice (Fig. 1C). As compared with respective WT subjects, mean Cyp2c KO responses were reduced after the larger, but not after the smaller icv dose of morphine (Fig. 1C). ANOVA (Fig. 1C, between groups [factor #1]: genotype; [factor #2]: dose; within groups [factor #3, repeated measures]: time) found significant main effects of time ($F_{4,96} = 56.9$, $P < 0.001$) but no significant terms related to genotype or dose of morphine. AUC analysis of Fig. 1C data found 28% and 0% reductions in mean KO vs. WT responses following 10 and 2 μg of morphine, respectively (brackets in 1C). However, genotype-related terms in the ANOVA of the icv AUCs did not reach statistical significance ($P=0.067$ for main effect of genotype).

Normal analgesic responses in Cyp2c KO mice following intrathecal morphine administration. Morphine was also administered into the spinal subarachnoid space of WT and Cyp2c KO mice (Supplemental Figure 1). Morphine (5 μg , intrathecal) produced highly effective analgesia in both genotypes. ANOVA (Supplemental Figure 1, between groups [factor #1]: genotype; within groups [factor #2, repeated measures]: time) only found significant main effects of time ($F_{5,50} = 21.7$, $P < 0.001$) with no significant terms related to genotype.

Normal $^3\text{HCIM}$ binding values in liver and brain from P450 gene cluster KO mice. $^3\text{HCIM}$ binding was studied in the P450 gene cluster KO mice in an attempt to identify cimetidine-binding P450 isoforms. Liver homogenates from Cyp2c, Cyp2d and Cyp3a KO

mice yielded specific $^3\text{HCIM}$ -binding values which were nearly identical with those from WT subjects (Supplemental Figure 2). One-factor (genotype) ANOVA of these data found no significant genotype differences ($P > 0.05$) in $^3\text{HCIM}$ binding. Binding values from brain homogenates of the four genotypes were very low (less than half of the magnitude found in rat brain), and were also not significantly different from each other (data not shown).

Discussion

The plethora of murine P450 genes (Nelson, et al., 2004) and the extremely low levels of P450s in brain (Iliff, et al., 2009; Ferguson and Tyndale, 2011) are formidable obstacles to identifying CNS roles for specific isoforms. However, the clustering of multiple, closely-related P450 genes made it possible to delete many genes from a P450 subfamily in single KO models (Scheer, et al., 2012a, 2012b; Hasegawa, et al., 2011; van Herwaarden, et al., 2007). The Cyp2c KO, Cyp2d KO, and Cyp3a KO mice tested have full-length deletions of 14, 9 and 7 P450 genes, respectively. Thus, the present work screened 30 murine P450 isoforms as potential $^3\text{HCIM}$ -binding proteins and as potential transduction elements of opioid analgesic signaling.

Characterization of $^3\text{HCIM}$ -binding proteins suggested a P450 profile, but these proteins were never identified (Stadel et al., 2008). Since inhibitors of $^3\text{HCIM}$ binding also block the effects of several types of analgesics, $^3\text{HCIM}$ -binding proteins were suggested to be targets for analgesic drug development (Hough, et al., 2007; Stadel, et al., 2010). The absence of deficits in $^3\text{HCIM}$ binding in liver homogenates from the genotypes studied (Supplemental Figure 2) demonstrates that none of 30 P450 isoforms deleted presently contribute to $^3\text{HCIM}$ binding.

The hypothesis that P450-catalyzed neuronal epoxygenase activity is required for μ opioid analgesia originated following discovery of attenuated analgesic responses to morphine in BCPRN mice (Conroy, et al., 2010). The defective analgesia was not explained by genotype differences in brain morphine levels or by differences in μ opioid receptor properties or signaling

(Conroy et al., 2010). Furthermore, P450 inhibitors and epoxygenase inhibitors antagonize morphine analgesia (Conroy, et al., 2010, 2013). Consistent with the hypothesis, Zhang and Pan (2012) showed that the in vitro effects of μ (but not δ) opioids in the brain stem are blocked by a P450 inhibitor, but the relevant isoforms are unknown. The present results (Fig. 1A), showing normal responses to morphine in the *Cyp2d* KO and *Cyp3a* KO mice, imply no roles for 16 gene products (Scheer, et al., 2012b; Hasegawa, et al., 2011) as mediators of μ opioid responses.

Since BCPRN mice (lacking neuronal P450 enzyme function) show defective morphine responses (Conroy et al., 2010), it was presently theorized that the morphine-relevant P450 isoforms might be discovered by demonstrating a deficit in the relevant KO mouse. The blunted maximal effect and shortened duration of action of s.c.-administered morphine in *Cyp2c* KO mice (Fig. 1A,B) seemed to support the possible relevance of CYP2C isoforms. Of particular mechanistic relevance might be CYP2C29, CYP2C37, CYP2C38 and CYP2C40, which are absent in the *Cyp2c* KO mice, but are normally expressed in the murine CNS, and are thought to have epoxygenase activity (Ilf, et al., 2009). However, since systemically-administered opioids act in the brain and spinal cord (Heinricher and Ingram, 2008), no CNS role for *Cyp2c* gene products in opioid analgesia was confirmed when morphine was given by icv (Fig. 2) or intrathecal (Supplemental Figure 1) dosing. Since synergistic interactions are known to occur between supraspinal and spinal opioid mechanisms (Yeung and Rudy, 1980), a role for the *Cyp2c* gene products in these interactions remains possible.

Fig. 2 shows a side-by-side comparison of the deficits in morphine analgesia seen presently in *Cyp2c* KO mice vs. earlier findings with BCPRN mice. Although statistically-significant reductions were seen in both mutants following s.c. morphine (41% and 54% reductions, respectively), BCPRN mice also showed a statistically-robust (38%) reduction in responses

following icv morphine vs. a non-significant tendency (28%) toward reduced responding in Cyp2c KO mice. Since BCPRN mice have a neuronal defect in cytochrome P450 reductase (required for all microsomal P450 activity), they are presumed to have reduced activity of many P450 isoforms. Although the non-significant tendency toward reduced responding in Cyp2c KO mice prevents strong conclusions, this deficit could imply the importance of multiple P450 subfamilies, including CYP2C. Alternatively, the significant reductions in analgesia seen in Cyp2c KO mice after s.c. but not icv morphine might be explained by decreases in plasma levels of morphine in the KO mice after s.c. administration. Since morphine is metabolized in mice by glucuronidation, and not by P450 mechanisms (Kuo, et al., 1991), Cyp2c KO mice could have adaptive increases in peripheral glucuronidation of morphine, thereby lowering plasma morphine levels. This has not been tested. Unlike the case for humans, mice do not have active morphine glucuronide metabolites (Kuo, et al., 1991). Unlike the discrepant findings with icv morphine (Cyp2c KO vs. BCPRN, Fig. 2), no genotype differences in analgesia were seen following intrathecal opioids in either Cyp2c KO (Supplemental Figure 1) or BCPRN mice (Hough et al., 2015). This pattern is consistent with a P450 role for supraspinal, but not spinal opioid mechanisms.

As discussed, the present results exclude 16 P450 isoforms from the 3a and 2d subfamilies as mediators of opioid analgesia, and four 2c isoforms may be important but require further study. Among these subfamilies, *Cyp3a13* (Hasegawa, et al., 2011) and *Cyp2c44* (Scheer, et al., 2012a) gene products were not studied, since these genes were not deleted in the present KO mice. The latter is a well known epoxygenase which deserves further study (Capdevila et al., 2007). It is clear that many additional P450 isoforms remain to be studied in the context of opioid analgesia. Among these, members of the CYP4X (Iliff, et al., 2009) and CYP2J (Graves, et al., 2013)

subfamilies have epoxygenase activity and are found in the CNS. Although the P450 epoxygenase hypothesis has not been confirmed, new classes of analgesic or anti-hyperalgesic medications are already being developed to mimic the actions of lipid epoxides (Brostram and Falck, 2011) or to inhibit their metabolism (Wagner, et al., 2013). The identification of the specific opioid-relevant P450 isoforms in the brain and their analgesic products will catalyze the development of novel pain relievers.

Authorship Contributions

Participated in research design: Hough

Conducted experiments: Nalwalk

Contributed new reagents or analytic tools: Scheer

Performed data analysis: Nalwalk, Hough

Wrote or contributed to the writing of the manuscript: Hough, Nalwalk, Ding, Scheer

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Footnotes

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Figure Legends

Figure 1. Morphine antinociception in P450 KO mice. A) Control (WT) and three lines of P450 gene cluster KO mice were baseline tested for nociceptive latencies (zero time), received morphine sulfate (20 mg/kg, s.c.) and were re-tested at the indicated post-injection times (abscissa, min). Nociceptive latencies (s, mean \pm SEM, ordinate) are shown for the number of subjects designated in parentheses. *,**P < 0.05, 0.01 respectively vs WT at the same time. B) Morphine antinociception in WT and Cyp2c KO mice shown as in A after two doses of morphine (10 and 20 mg/kg, s.c.). Data from 20 mg/kg group are re-drawn from A. Areas under the curves (AUCs, mean \pm SEM, s, 30-120 min) are given in brackets for the four groups. *ANOVA of latencies found significant main effect (P<0.05) of genotype. C) WT and Cyp2c KO mice were baseline tested (zero time), received the indicated dose of morphine sulfate (icv) and were re-tested at the designated post-injection times (abscissa, min). Latencies (s, mean \pm SEM, ordinate) are shown for the number of subjects in parentheses. AUCs are given in brackets (mean \pm SEM, s, 20-180 min) for the four groups. No genotype differences were detected after icv morphine.

Figure 2. Deficits in morphine analgesia in two genotypes of P450-deficient mice. Responses in Cyp2c KO mice are compared with those found in Brain CPR Null (BCPRN) mice, a conditional KO lacking microsomal brain neuronal P450 activity. Latencies (ordinates, mean \pm SEM) are shown for each treatment for the number of subjects in parentheses. A) Effects of s.c.-administered morphine in Cyp2c KO and WT control mice (left 2 bars, data are 60 min after 10 mg/kg, s.c., from Fig. 1B) vs. BCPRN and WT mice (right 2 bars, data are 90 min after 20 mg/kg, s.c., taken from Hough, et al., 2014). B) Effects of icv-administered morphine (60 min

after 10 μ g) in Cyp2c KO and WT control mice (left 2 bars, data from Fig. 1C) and in BCPRN and WT mice (right 2 bars, data from Hough, et al., 2014). Downward arrows show mean percent reductions in responses in each mutant genotype vs. respective WT controls. **BCPRN responses significantly different ($P < 0.01$) vs. WT by ANOVA and post-hoc comparisons. +Significant ($P < 0.05$) genotype difference revealed by ANOVA, but not significantly different at this time by post-hoc testing.

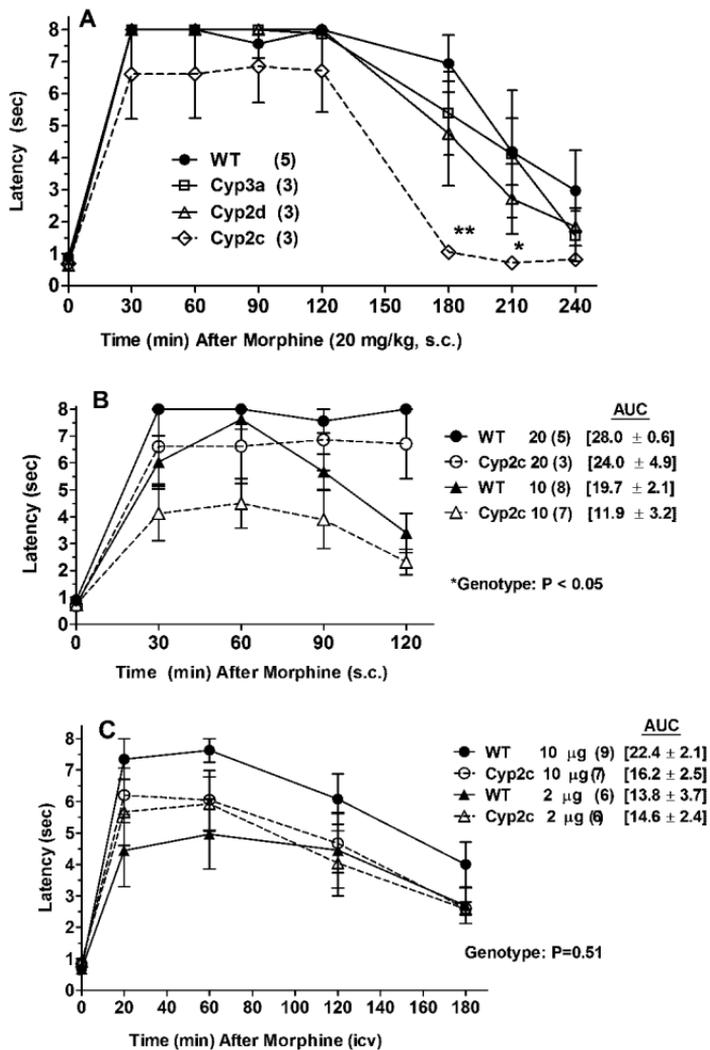


Figure 1

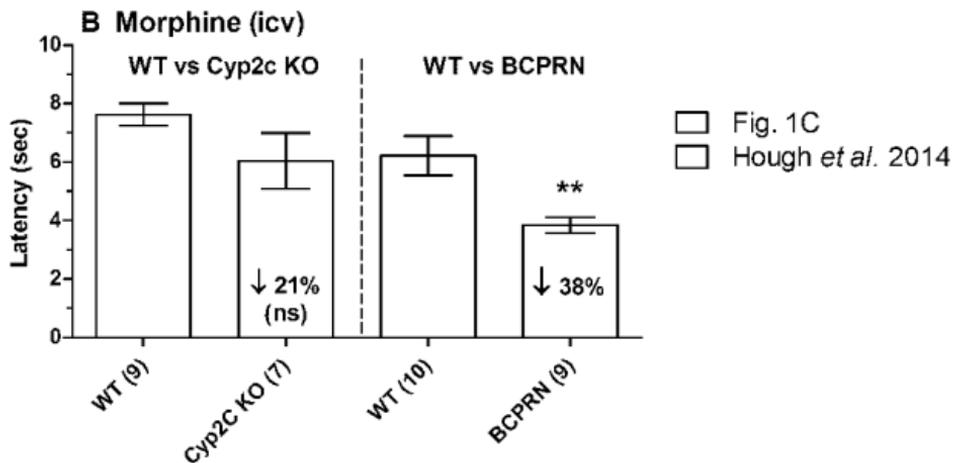
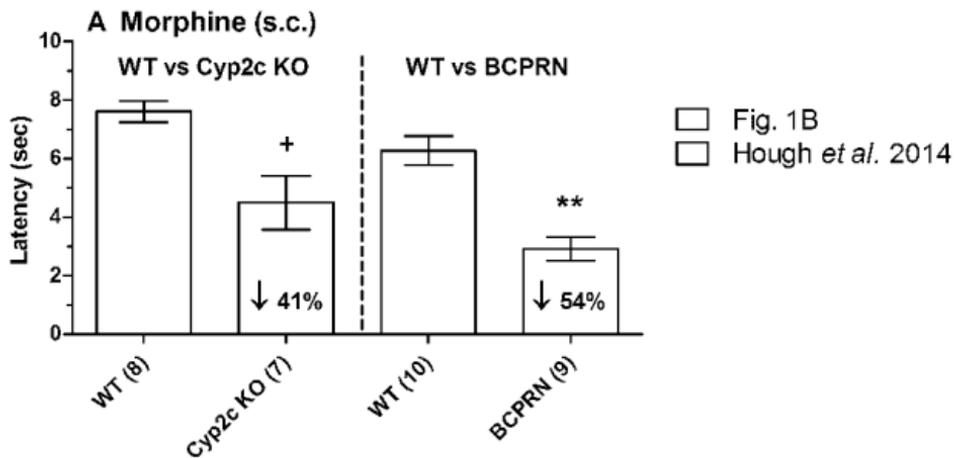


Figure 2

Short Communication

Drug Metabolism and Disposition

Opioid Analgesia in P450 Gene Cluster Knockout Mice:

A Search for Analgesia-Relevant Isoforms

Supplement

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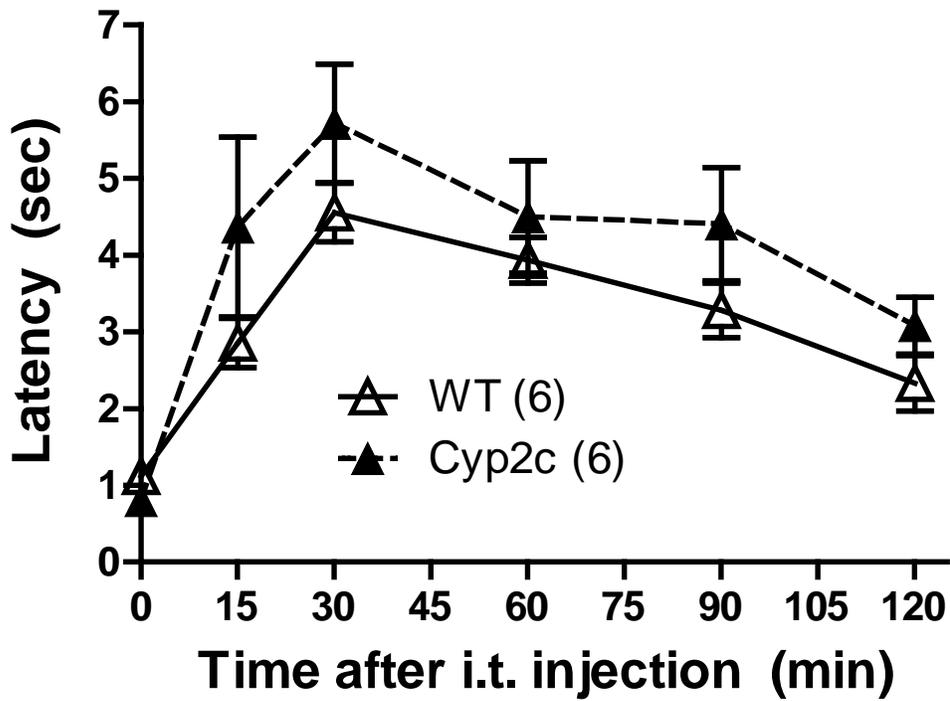
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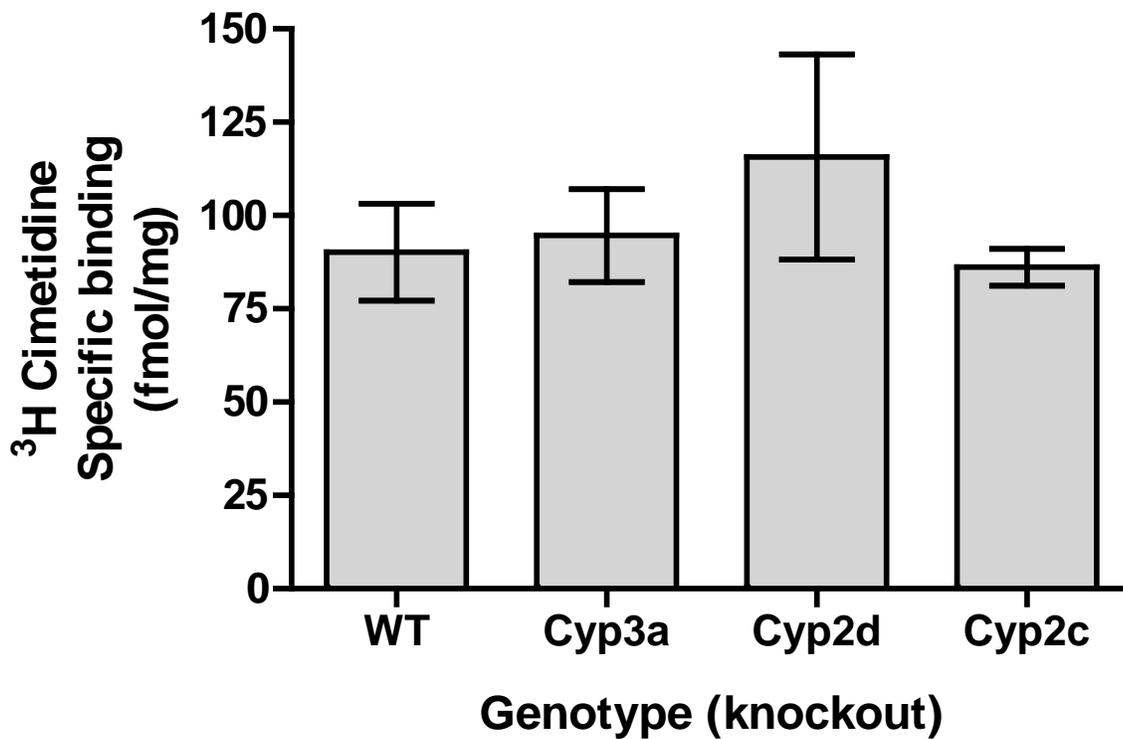
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Supplement Figure 1. Intrathecal (i.t.) morphine antinociception in Cyp2c KO mice. Control (WT) and KO mice were baseline tested (zero time), received morphine sulfate (5 μ g) and were re-tested at the designated post-injection times (abscissa, min). Latencies (sec, mean \pm SEM, ordinate) are shown for the number of subjects in parentheses.



Supplement Figure 2. ³H-Cimetidine binding in liver homogenates. Membrane fractions from homogenates of control (WT) and three genotypes of P450 gene cluster KO mice were incubated with ³H-cimetidine, filtered and counted as described. Specific binding values (fmol/mg, ordinate, mean \pm SEM) are shown for livers from three subjects (each determined in triplicate) of each genotype.