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Ethanol Reversal of Tolerance to the Antinociceptive Effects of Oxycodone and Hydrocodone

by

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Abstract

This study compared the development of tolerance and its reversal by ethanol of two orally-bioavailable prescription opioids, oxycodone and hydrocodone, to that of morphine. Oxycodone (s.c) was significantly more potent in the mouse tail withdrawal assay than either morphine or hydrocodone. Oxycodone was also significantly more potent in this assay than hydrocodone when administered orally. Tolerance was seen following chronic subcutaneous administration of each of the three drugs and by the chronic administration of oral oxycodone, but not following the chronic oral administration of hydrocodone. 1 g/kg i.p. ethanol significantly reversed the tolerance that developed to the subcutaneous administration of each of the three opioids. It took twice as much ethanol when given orally to reverse the tolerance to oxycodone. We investigated whether the tolerance observed to oxycodone and its reversal by ethanol were due to bio-dispositional changes or were reflecting a true neuronal tolerance. As expected, a relationship between brain oxycodone concentrations and activity in the tail immersion test existed following administration of acute oral oxycodone. Following chronic treatment, brain oxycodone concentrations were significantly lower than acute concentrations. 2 g/kg oral ethanol reversed the tolerance to chronic oxycodone, but did not alter brain concentrations of either acute or chronic oxycodone. These studies show that there is a metabolic component of tolerance to oxycodone, however the reversal of that tolerance by ethanol is not due to an alteration of the bio-disposition of oxycodone, but rather is neuronal in nature.

Introduction

Prescription opioids such as oxycodone were responsible for over half of the reported 28,000 opioid overdose deaths in 2014 (CDC, 2016). Individuals who abuse prescription opioids often use other substances leading to poly-drug abuse (Ogbu *et al.*, 2015). Ethanol is one of the most commonly co-abused drugs by opioid users, despite the long-standing warning that ethanol and opioids pose a significant health risk when taken together (Karch and Drummer, 2001; Oliver *et al.*, 2007). Multiple post-mortem analyses have shown that individuals who consumed opioids such as heroin along with alcohol died from blood opioid concentrations measuring significantly below than those who died from an opioid without alcohol consumption (Darke and Hall, 2003). Additionally, a separate study that specifically investigated oxycodone-related overdoses reported that deaths resulting from the combined intake of oxycodone and ethanol were ruled to be exclusively accidental, rather than intentional (Thompson *et al.*, 2008). While it is possible that a general lack of awareness exists among opioid users regarding the dangers related to co-consumption of ethanol with opioids, it is likely that these individuals experience some enhancement of opioid effects when used together, leading to riskier drug taking behavior to offset the tolerance(s) developed to the opioids. Post-mortem studies reported findings from blood levels extracted from peripheral sites such as the femoral artery and heart blood. Collection of post-mortem blood is easy to obtain and samples are reliably quantified, however it is important to recognize that the lethal event during opioid overdose is respiratory depression – a centrally-mediated effect controlled primarily in the brainstem where opioid receptor density is quite high (Delfs *et al.*, 1994; Satoh and Minami, 1995). One hypothesis relating to the increased lethality of oxycodone when ethanol was also detected in presumed opioid-tolerant individuals, is that ethanol is altering the kinetics of oxycodone. Therefore, it was important to investigate if in fact the distribution and concentration of oxycodone in the brain is altered by ethanol administration.

Ethanol is also known to reverse various tolerances to morphine, including the antinociceptive and respiratory depressive effects, which may be explained by mechanisms that involve PKC and $GABA_A$ and $GABA_B$ receptor signaling (Hull *et al.*, 2013; Hill *et al.*, 2016). Additionally, recent evidence has shown that ethanol was unable to reverse the respiratory depressive tolerance to methadone, suggesting that certain opioids may be more susceptible to ethanol's reversal effects than others (Withey *et al.*, 2017). Our goal for the studies presented here was to compare the effects of ethanol reversal as seen previously in morphine tolerant mice to those of mice made tolerant to two other commonly abused opioids, oxycodone and hydrocodone, to determine if they are more "morphine-like" or more "methadone-like" in regards to their interaction with ethanol.

These drugs, like morphine and heroin, have been shown to exert their analgesic and respiratory depressive effects through similar pathways and mechanisms involving the μ-opioid receptor. There are a number of unique properties belonging to oxycodone and hydrocodone, however, that set them apart from morphine beyond slight structural differences, such as varying degrees of oral bioavailability and their primary enzymatic degradation pathways (Reisine and Pasternak, 1996; Kolesnikov *et al.*, 2003). Discrepancies exist in the literature regarding which opioid receptor mediates oxycodone's antinociceptive effects, with studies carried out in rats supporting a primary role of the kappa receptor based on *in vitro* binding studies and behavioral assessments (Nielsen *et al.*, 2007). Studies utilizing *in vitro* and *in vivo* approaches in mice however, showed the μ-opioid receptor is the primary receptor type that is preferentially bound and activated by oxycodone (Yoburn *et al.*, 1995). Additionally, it was shown that in the tail flick assay, neither NorBNI nor naltrindole, the k opioid receptor and δ opioid receptor antagonists respectively, were unable to block the antinociceptive properties of oxycodone (Beardsley *et al.*,

2004). Given the differences underlying the pharmacodynamics and pharmacokinetics of these opioid compounds, it was of interest to determine if ethanol reversed analgesic tolerance to both of these compounds, and to what degree each of the compounds were susceptible to ethanol's reversal effects.

Materials and Methods

Drugs and Chemicals. Morphine sulfate, Oxycodone HCl, Hydrocodone bitartrate and 75-mg morphine pellets and placebo pellets were obtained from the National Institutes of Health National Institute on Drug Abuse (Bethesda, MD). Morphine sulfate, Oxycodone HCl and Hydrocodone bitartrate were each dissolved in pyrogen-free isotonic saline (Hospira, Lake Forest, IL). Ethanol was obtained from AAPER Ethanol and Chemical Co. (Shelbyville, KY) and was diluted with pyrogen-free isotonic saline.

Animals. Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25–30 g were housed five to a cage in animal care quarters and maintained at 22 ± 2°C on a 12-hour light-dark cycle. Food and water were available ad libitum. The mice were brought to the test room (22 ± 2°C, 12-hour light-dark cycle), marked for identification, and allowed 18 hours to recover from transport and handling. Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University Medical Center and comply with the recommendations of the International Association for the Study of Pain (IASP).

Tail Immersion Test. The warm-water tail immersion test was performed using a water bath with the temperature stabilized at 56 ± 0.1°C (Coderre and Rollman, 1983). Before injecting the mice, a baseline (control) latency was determined. Only mice with a control reaction time between 2 to 4 seconds were used. Test latencies were assessed 20 minutes following opioid treatment, with a 10-second maximum cut-off time utilized to prevent tissue damage. Antinociception was quantified as the percentage of maximum possible effect (%MPE), which was calculated as: %MPE = [(test latency – control latency) / (10 – control latency)] X 100. Percent MPE was calculated for each mouse using at least six mice per dose of drug (Harris and Pierson, 1964).

Acute Dose Response Curves in Tail Immersion Test: Oxycodone, Hydrocodone and Morphine. Male Swiss Webster mice were weighed and baseline tail withdrawal latencies were recorded as described above. Oxycodone was administered subcutaneously at doses of 0.25, 0.5, 1.0, 1.25 and 1.5 mg/kg and mice were returned to their home cage. Hydrocodone was administered subcutaneously at doses of 1.0, 3.0, 5.0 and 6.0 mg/kg and mice were returned to their home cage. Morphine was administered subcutaneously at doses of 2.0, 4.0, 8.0, and 16 mg/kg and mice were returned to their home cage. After a 20-minute pretreatment period, mice were re-tested for tail withdrawal latencies to assess antinociceptive effects and to conduct dose-response curves.

Single-Day Tolerance Model. Antinociceptive tolerance to oxycodone and hydrocodone was developed as follows. Mice were injected subcutaneously (s.c.) once every hour (for a total of seven injections) with the respective ED_{80} dose of each opioid corresponding to the tail immersion test, 1.25 mg/kg for oxycodone and 5.0 mg/kg for hydrocodone, and saline in control mice. One hour after the final injection, mice were administered 1 g/kg ethanol or 0.9% saline vehicle by intraperitoneal (i.p.) injection and 30 minutes later were challenged with various subcutaneous doses of oxycodone (ascending $\log_2$ doses from 0.25 – 4.0 mg/kg) or hydrocodone (ascending $\log_2$ doses from 1 – 16 mg/kg) to construct dose-response curves for calculation of ED_{50} values and potency ratios.

4-day Tolerance Model. Tolerance to oral oxycodone or hydrocodone was developed using a twice-daily gavage method whereby animals were administered 64 mg/kg oxycodone, or 128 mg/kg hydrocodone, in the morning and again in the evening, with at least 8 hours separating the two gavage events. Animals were weighed on day 1, 3 and 5 (test day) and dosing was adjusted accordingly. The evening gavage administration on day 4 was the final maintenance dose animals

received prior to test and challenge treatments on day 5. Drug dose was calculated for 0.1cc/10g body weight administration with 0.9% physiological saline as the vehicle. All mice had continued access to *ad libitum* food and water throughout the dosing paradigm and remained group-housed in their home cages.

Oral Oxycodone Time Course. A time course study was conducted to assess the antinociceptive effects of oral oxycodone following a single gavage of 16 mg/kg oxycodone at the following time points: 5, 10, 20, 30, 60, 120, 240 and 480 minutes. Here, 5 mice were repeatedly tested to determine average %MPE at each time point. Utilizing the same time points and dose of oxycodone, we repeated the time course study to assess brain oxycodone concentrations. An N of 5 mice per time point was utilized for the brain concentration analysis.

Reagents for GC/MS Analysis. The primary reference materials of morphine, morphine-d_3, oxycodone and oxycodone-d_6 were purchased from Cerilliant Corporation (Round Rock, Texas) as metabolic solutions. The chloroform, deionized (DI) water, hydroxamine hydrochloride (HCL), 2-propanol, sodium bicarbonate and sodium carbonate were purchased form Fisher Scientific (Hanover Park, Illinois). BSTFA (N,O-bis(trimethylsilyl)-trifluoroacetamide) + 10% TMCS (Trimethylchlorosilane) was purchased from Regis Technologies (Morton Grove, Illinois).

Sample Extraction. Quantitative analysis of morphine and oxycodone was based upon a previously described method (Broussard *et al.*, 1997; Wolf and Poklis, 1997). This method is routinely preformed in our laboratory for the analysis of opiates in blood and tissue samples. Pre-extraction preparation was unnecessary for the whole blood specimens. Whole brain tissues specimens were diluted as 1 part tissue to 3 parts deionized water (v:v) and homogenized. Matched matrix five-point calibration curve containing opiates of interest were prepared at 20-1000 ng/mL for blood or 20-1000 ng/g for tissue, along with a blank and a double blank controls. 10 µL of

internal standard (ISTD) consisting of 10 μg/mL (100 ng total) of morphine-d_3 and oxycodone-d_6 was added to 1.0 mL or 1.0 g aliquots of calibrators, controls and specimens, except the double blank control. 0.2mL of 10% hydroxamine HCL was added to each sample. They were then mixed and heated at 30°C for 30 mins. Samples were then cooled and 1 mL of saturated carbonate/ bicarbonate buffer (1:1, N:N, pH 9.5) and 2 mL of chloroform:2-propanol (8:2) were added. Samples were mixed for 5 min and then centrifuged at 2500 rpm for 5 min. The top aqueous layer was aspirated and the organic layer was transferred to a clean test tube and evaporated to dryness at 40°C under a constant stream of nitrogen. 50 μL of BSTFA (N,O-bis(trimethylsilyl)-trifluoroacetamide) + 10% TMCS (Trimethylchlorosilane) was added and the samples were heated for 30 min at 70°C. The samples were then placed in auto-sampler vials for gas chromatography mass spectrometry (GC/MS) analysis.

Instrumental Analysis. The GC/MS analysis was performed with a Hewlett-Packard 6890 with a split/splitless injection port attached to Hewlett-Packard model 5793A mass selective detector (MSD) with a 7683 autosampler. The chromatographic separation was preformed on an Agilent (Santa Clara, California) HP-1 12 m x 0.2mm x 0.33 μm analytical column with the injection temperature set to170°C in run in pulsed splitless mode. The initial oven temperature was 170°C and was held for 1.0 min., then heated at 10°C/min. to 280°C. The total run time was 12 min. The quantification and qualifying ions monitored for were for morphine 236, 414 and 429 *m/z*; for oxycodone 269, 459 and 474 *m/z*; for morphine-d3 239 and 432 *m/z*; and for oxycodone-d_6 465 and 480 *m/z*. A linear regression of the ratio of the peak area counts of quantification ion of ISTD versus concentration was used to construct the calibration curves.

Data Analysis. Opioid dose response curves were conducted for calculation of ED_{50} values by the method of Bliss (1967), utilizing least-square linear regression analysis followed by

calculation of 95% confidence limits (Bliss, 1967). For all other statistical analyses, GraphPad Prism 5 was used (GraphPad Software, Inc., La Jolla, CA). All data are represented as mean ± standard error of the mean. A One-way ANOVA with Tukey's post-hoc analysis was utilized when comparing changes across three or more groups over a single factor. Statistical differences between only two groups of data were analyzed using Student's two-tailed unpaired t-test. Significant differences were considered when $P < 0.05$.

Results

Acute Effects of Oxycodone and Hydrocodone.

To test acute antinociceptive properties, oxycodone or hydrocodone was administered subcutaneously and assessed in the warm water tail withdrawal test. Tail withdrawal latencies dose-dependently increased, reaching a ceiling effect at 1.5 mg/kg for oxycodone and at 6.0 mg/kg for hydrocodone, where all animals exhibited an MPE of 100%. The ED_{50} value for acute oxycodone was calculated to be 0.84 mg/kg (0.68 – 1.04). The ED_{50} value for hydrocodone was calculated to be 3.95 mg/kg (2.40 – 6.52). Morphine was administered subcutaneously with all mice reaching 100% MPE at 8 mg/kg. The ED_{50} was calculated to be 3.94 mg/kg (3.55 – 4.36). As predicted, we found that oxycodone was more potent than hydrocodone, which was equally as potent as morphine when administered subcutaneously in mice.

We then compared the potencies of oxycodone and hydrocodone when given orally, due to their reliable oral bioavailability. Acutely, oxycodone produced an oral ED_{50} of 9.29 mg/kg (7.18 – 12.02), ten times its subcutaneous ED_{50} value. In subsequent experiments comparing chronic saline treated (i.e. control) versus chronic oxycodone treated mice, a similar ED_{50} value of 8.29 mg/kg (6.12 – 11.52) was obtained in the control mice, indicating acute oral oxycodone ED_{50} values are reliably reproducible (Table 1). Hydrocodone orally produced an ED_{50} value also nearing ten times the subcutaneous value, equaling 38.79 mg/kg (29.21 – 51.53) (Table 1).

Tolerance Developed to Oxycodone and Hydrocodone Following Repeated Administration.

Oxycodone Tolerance: Chronic injections of s.c. saline prior to s.c. oxycodone challenge doses yielded a dose response curve of oxycodone with an ED_{50} value of 0.90 mg/kg (0.72 - 1.12), reproducing what was observed in our acute dose response experiments, suggesting no adverse effects of handling or repeated vehicle injections on oxycodone's antinociceptive effect. In mice

repeatedly administered s.c. oxycodone prior to receiving s.c. oxycodone challenge doses, the ED_{50} value was 1.70 mg/kg (1.42 - 2.03), significantly shifted to the right compared to the mice that received chronic saline, indicating tolerance was observed (Figure 1A). We further characterized oxycodone tolerance development by investigating the response to repeated oral oxycodone. A modified protocol with repeated exposure across 4 days, rather than repeated exposure within a single day, was utilized for this experiment. We found a significant shift to the right in response to chronic oral oxycodone with an ED_{50} value of 33.41 mg/kg (25.50 – 44.17) compared to the acute ED_{50} value of 8.29 mg/kg (6.12 -11.52) reported above, demonstrating that these mice were tolerant to the antinociceptive effects of orally administered oxycodone.

Hydrocodone Tolerance: Chronic s.c. injections of saline followed by acute challenge doses of s.c. hydrocodone generated a dose response curve with an ED_{50} value of 3.92 mg/kg (3.26 -4.71) (Figure 1B) in mice, similar to what was observed in previous acute dose response experiments as stated above. The ED_{50} value significantly shifted to the right in animals chronically injected with hydrocodone prior to receiving the challenge injections, equaling 9.01 mg/kg (6.44 – 12.62) and indicating tolerance developed in these mice. We also investigated the development of tolerance to oral hydrocodone and utilized a 4-day protocol similar to what was used for our chronic oral oxycodone studies. We observed an increase in the oral ED_{50} value, equaling 55.92 mg/kg (40.63 – 76.96), however, the confidence limits overlap that of the acute oral ED_{50} value (38.79 mg/kg (29.21 – 51.53)), suggesting that complete tolerance was not observed in the oral dosing study (Table 1). In a follow-up study, we increased the maintenance dose to 256 mg/kg po and still did not observe tolerance to the antinociceptive effects of hydrocodone (data not shown).

Reversal of Oxycodone and Hydrocodone Antinociceptive Tolerance by Ethanol (i.p.). We tested 1 g/kg ethanol i.p. administration in mice repeatedly administered oxycodone or

hydrocodone. Previously, our lab determined that a dose of 1g/kg ethanol was inert in the warm water tail withdrawal test, but fully reversed morphine tolerance in mice (Hull et. al, 2013). ED_{50} values were calculated from the resulting dose response curves and compared across three conditions: chronic saline followed by saline and acute opioid challenge, chronic opioid followed by saline and opioid challenge, and chronic opioid followed by ethanol and opioid challenge. A single injection of 1g/kg ethanol reversed antinociceptive tolerance to both oxycodone and hydrocodone (Figure 1A and 1B) as shown by the restoration of the ED_{50} values which closely resembled values observed in the acute dose response experiments (Table 1). These results add to our previous findings that ethanol reversed the analgesic tolerance to morphine, suggesting that ethanol may be interfering with a pathway common to some, but not all, opioids.

Reversal of Oxycodone Antinociceptive Tolerance by Oral Ethanol. Mice were assessed for oxycodone tolerance and reversal by po ethanol in addition to ip ethanol, again utilizing the single-day tolerance paradigm. Mice that were repeatedly injected with s.c. oxycodone and received a saline gavage displayed tolerance to the antinociceptive effects in response to a 1.25 mg/kg oxycodone s.c. challenge injection as shown by a significantly lower %MPE (10.25% ± 2.71) ($P < 0.05$, One-way ANOVA) when compared to acute oxycodone controls (50.98% ± 11.95). Mice that were repeatedly injected with s.c. oxycodone but received a 2 g/kg po ethanol gavage prior to receiving the challenge s.c. oxycodone injection continued to respond to oxycodone, displaying antinociceptive responses similar to that of both acute oxycodone treated mice and oxycodone-treated mice that received 1 g/kg ethanol i.p. The %MPE values observed in both of these chronic oxycodone plus ethanol treatment groups displayed significantly higher %MPE values compared to that of the chronic oxycodone plus saline treatment group, $P < 0.05$ (2 g/kg ethanol po, 63.90%

± 16.88) and $P < 0.01$ (1 g/kg ethanol ip, 71.49% ± 11.83), (One-way ANOVA) (Figure 2). These data indicate that both i.p. and p.o. ethanol reversed oxycodone tolerance.

Pharmacokinetic Studies

Acute Oral Oxycodone Time Course: Antinociception and Brain Concentrations. 16 mg/kg p.o. oxycodone was detectable in the brain at the earliest time point measured of 5 minutes, averaging 80.2 ng/g, while antinociception was marginal at 10.14% MPE on average. Peak brain concentrations were observed at 20 and 30 minutes following oxycodone administration, 153.58 ng/g and 153.24 ng/g respectively. Brain concentrations at these times were significantly higher than concentrations detected at 120 minutes ($P < 0.05$) and 480 minutes ($P < 0.001$) (One-way ANOVA). Significant antinociception was detected at the 20, 30 and 60 minute time points but not at the 5 minute time point (($P < 0.05$ (20 min) and $P < 0.001$ (30, 60 min), One-way ANOVA). Peak antinociception, measured as 100% MPE, was not detected until 30 minutes following administration, and persisted until the 60 minute point. Antinociception at 20 ($P < 0.05$), 30 and 60 minutes following oxycodone was significantly higher than 480 minutes ($P < 0.0001$), with the 30 and 60 minute time points also significantly higher than at 240 minutes ($P < 0.001$, One-way ANOVA) Brain oxycodone concentrations at 60 minutes however were markedly lower, measuring on average closer to the 5 and 10 minute time points, though the antinociceptive effects were vastly greater. Notably, oxycodone was not detectable in any of the brain tissue samples tested 480 minutes (8 hours) after a single gavage and there was no antinociception at this time (Figure 3).

Brain Oxycodone Concentrations Did Not Correlate With Antinociception. We compared brain concentrations of oxycodone when given orally versus subcutaneously using equianalgesic doses. 1.25 mg/kg s.c. and 16 mg/kg p.o. oxycodone both produced near 80% MPE. 1.25 mg/kg

s.c. oxycodone produced 74.48% MPE while 16 mg/kg po oxycodone produced 77.71% MPE. Brain oxycodone concentrations averaged 348.89 ng/g following subcutaneous administration, significantly higher than oral oxycodone concentrations which averaged 114.1 ng/g ($p < 0.0001$, Student's two-tailed unpaired t-test). Our findings indicate that the antinociceptive effects of oxycodone are not directly correlated with brain oxycodone concentrations (Figure 4).

Acute Brain Concentrations of Oxycodone After Oral Administration. Oxycodone was administered orally in ascending $\log_2$ doses ranging from 8 to 64 mg/kg to assess a dose response relationship for brain concentrations. Brain concentrations showed an average of 137.2 ng/g (N = 5), 114.1 ng/g (N = 10), 312.4 ng/g (N = 5), and 731.2 ng/g (N = 14) 20 minutes following administration of 8, 16, 32 and 64 mg/kg oxycodone respectively. Significant differences were detected between the 64 mg/kg and 16 mg/kg doses ($P < 0.01$, One-way ANOVA), as well as between 64 mg/kg and the 8 mg/kg dose ($P < 0.05$, One-way ANOVA).

Brain Concentrations of Oral Oxycodone After Repeated Administration. The development of oral oxycodone tolerance was carried out over 4 days via twice daily gavage administrations of 64 mg/kg oxycodone. Mice were challenged on day 5 with a single gavage of 16 mg/kg oxycodone. Brain concentrations 20 minutes following the challenge gavage showed an average of 28.92 ng/g (N = 13). These concentrations were significantly lower than seen after acute 16 mg/kg at the 20 minute time point ($P < 0.0001$) (Figure 5A).

The Effect of Oral Ethanol on Brain Concentrations of Acute Oral Oxycodone. To determine if ethanol alters acute oxycodone brain concentrations, mice were pretreated with 2 g/kg ethanol 30 minutes prior to receiving a gavage of 16 mg/kg oxycodone. Mice were sacrificed 20 minutes following oxycodone administration and brain samples were collected and processed immediately thereafter. After the oral administration of 2 g/kg ethanol, brain concentrations averaged 304.7 ±

90.8 ng/g. Brain oxycodone concentrations were not significantly altered by 2 g/kg acute ethanol as compared to acute oxycodone alone ($P > 0.05$, Student's two-tailed t-test) (Figure 5B).

The Effect of Ethanol on Chronic Oxycodone Brain Concentrations. To determine if ethanol altered chronic oxycodone brain concentrations, mice were repeatedly administered oxycodone twice daily for four days and pretreated on day 5 with a gavage of 2 g/kg ethanol 30 minutes prior to receiving a challenge gavage of 16 mg/kg oxycodone. Mice were sacrificed 20 minutes following oxycodone administration and brain samples were collected and processed immediately thereafter. Brain oxycodone concentrations following chronic oxycodone administration and the acute administration of 2 g/kg ethanol equaled 26.13 ± 3.45 ng/g (N = 8) (Figure 5C). Chronic oxycodone concentrations measured in the presence of ethanol were consistent with the chronic oxycodone samples measured in the absence of ethanol, providing supporting evidence that acute ethanol did not alter chronic oxycodone brain concentrations ($P > 0.05$, unpaired Student's two-tailed t-test).

Discussion

Oxycodone and hydrocodone are two of the most commonly prescribed opioids for the relief of pain despite their untoward side effects including tolerance development and abuse liability. Presently, an opioid abuse epidemic exists, in part, due to numerous individuals becoming dependent on prescription opioids, who then switch to heroin. When alcohol is consumed simultaneously with heroin, the risk of overdose and death increases. This could be due to one drug potentiating or adding to the depressant effects of the other, or due to the reversal of the tolerances that have developed to the opioid. We have previously shown that ethanol reversed morphine tolerance. The goal of this study was to compare the acute potency, propensity to produce tolerance, and assess reversal of that tolerance to oxycodone and hydrocodone by ethanol. A second goal was to elucidate if the tolerance was reversed due to an alteration of pharmacokinetic parameters.

Characterization of the Development of Oxycodone and Hydrocodone Antinociceptive Tolerance and the Effect of Ethanol on that Tolerance. Using a single-day injection schedule, mice were made tolerant to either oxycodone or hydrocodone. We found a 2-fold rightward shift in ED_{50} values in mice repeatedly injected with oxycodone. The doses we used produced similar levels of antinociception as seen in other studies, though a different outbred mouse strain (ICR) was used (Beardsley *et al.*, 2004; Minami *et al.*, 2009). Similarly, we found a 2-fold rightward shift in ED_{50} values in mice repeatedly injected with hydrocodone. Interestingly, our acute ED_{50} values for hydrocodone are between values reported by two others studies that used the radiant heat tail flick assay rather than warm-water tail immersion. Kolesnikov *et al.*, (2003) found an ED_{50} value of 1.37 mg/kg in Swiss Webster mice bred by a different vendor, while Navani and Yoburn (2013), calculated an ED_{50} value of 11 mg/kg in CD-1 mice.

Ethanol was similarly effective in reversing the antinociceptive tolerance to both oxycodone and hydrocodone when administered at 1 g/kg i.p. In previous studies, this dose was also effective at reversing analgesic tolerance and respiratory depressive tolerance to morphine (Hull *et al.*, 2013; Hill *et al.*, 2016). Further, 20mM *in vitro* ethanol reversed *ex vivo* morphine tolerance in the locus coeruleus of rats, suggesting that low-to-moderate ethanol doses reverse tolerance without eliciting effects acutely (Llorente *et al.*, 2013).

Pharmacokinetic Analysis of Acute Oxycodone Time Course. We investigated the relationship between brain oxycodone concentrations and antinociceptive effects in mice. Previously, we have shown that there is a correlation between brain morphine concentrations and tail flick latencies (Patrick *et al.*, 1975, 1978). Oxycodone was detected in brain tissue 5 minutes after the oral administration of 16 mg/kg, but antinociception was minimal. The concentration continued to increase until 20 minutes post gavage, where it remained steady for at least ten more minutes, indicating peak concentrations for this dose of oxycodone were present in the brain. Significant antinociception was observed 20 minutes following oxycodone administration, with maximal or near maximal effects lasting from 20 to 60 minutes. Given that oxycodone was administered orally, it is not surprising that peak concentrations were not detected sooner. Additionally, mice were not food restricted in these studies, and gastric emptying time could have altered or delayed the time to which oxycodone was actually absorbed and distributed through the liver and to the brain. In addition, at 60 minutes, oxycodone concentrations were lower, though not significantly, as compared to peak concentrations at 20 and 30 minutes. Between 30 minutes and 60 minutes, there was a 50% decrease in oxycodone concentrations. The antinociceptive effects between 30 and 60 minutes however, did not change and continued to produce 100% MPE. This discrepancy between brain concentrations of oxycodone and the antinociceptive effect measured likely suggests the

presence of an active metabolite, however our study did not investigate which metabolite(s) contributed to our observation. Oxycodone was not detected in the brain 8 hours after mice were dosed and no antinociceptive effects were observed.

Brain Concentrations of Oxycodone After Chronic Administration. In chronic oxycodone treated-mice, brain oxycodone concentrations following a challenge gavage of 16 mg/kg were significantly lower compared to those observed in brains of mice that only received a single administration of the same dose of oxycodone. The effect was consistent, as demonstrated by the minimal variability between sample values, suggesting a well-regulated mechanism underlies oxycodone tolerance and metabolism. It is possible that while initial variances in individual response are likely to occur upon acute exposure, highly regulated signaling events and selectively activated enzymatic pathways lead to more structured biological responses upon repeated exposure. These data also provide further insight into the potential mechanisms underlying oxycodone tolerance. There could be a significant upregulation of degradative enzymes leading to the development of metabolic tolerance, and warrants further investigation into this possible explanation. Additionally, the reduction in brain oxycodone concentrations after repeated administration could be a result of increased P-glycoprotein (P-gp) activity, a chaperone protein that actively transports drug molecules across the blood brain barrier. P-gp is well characterized in its effects on opioid agonists (Dagenais *et al.*, 2004), yet there are opposing reports regarding P-gp's actions on oxycodone. One study showed P-gp's ATP-ase activity was dose-dependently increased by acute oxycodone and was upregulated after chronic oxycodone in rats (Hassan *et al.*, 2007), however, another study showed that the P-gp inhibitor, PSC833, had no effect on oxycodone's ability to enter and remain in the brain in rats (Bostrom *et al.*, 2005). The contributions of the latter study's findings are difficult to interpret given that PSC833 has been

shown to be less specific than previously thought (Mayer *et al.*, 1997; Cvetkovic *et al.*, 1999).

Acute and Chronic Oxycodone Pharmacokinetics Unaffected by Ethanol Co-administration in Mice. One of our main objectives in this study was to better characterize the effect of doses of ethanol that are moderately intoxicating in humans on acute and chronic brain oxycodone concentrations in mice. In our acute ethanol and acute oxycodone study we found that oral ethanol at a dose of 2 g/kg did not significantly alter oxycodone brain concentrations. It was of utmost importance to evaluate the effects of ethanol on brain oxycodone concentrations in chronic oxycodone treated mice in order to address the primary health concern of poly-drug abuse leading to opioid overdose. We tested 2 g/kg oral ethanol on chronic oxycodone brain concentrations. Somewhat unexpectedly, we observed that 2 g/kg ethanol did not significantly alter oxycodone brain concentrations. These results in brain tissue differ from blood results from multiple human post-mortem analyses, where the co-detection of ethanol corresponded with significantly lower opioid levels in the blood compared to those where only opioids were detected at time of death (Kerr *et al.*, 2007; Darke, 2011). These data do however agree with and add to the previous observations in Hill et al (2016) where 0.3 g/kg ethanol ip did not alter morphine brain or plasma concentrations in mice while still reversing the tolerance to respiratory depression, suggesting that ethanol is not working through mechanisms that alter the kinetics of either of these opioids (Hill *et al.*, 2016). Furthermore, our findings were the result of acute ethanol effects on oxycodone brain concentrations, whereas repeated ethanol treatments might alter those findings. The effects of repeated ethanol have been tested on morphine behavioral responses and [^{3}H]-dihydromorphine binding in mice, where a change in affinity for striatal opioid receptors was observed after ethanol feeding (Tabakoff *et al.*, 1981). Clearly, ethanol effects on opioid blood and brain concentrations in mice are dependent on treatment regimen and the specific opioid compound tested.

These studies show that there is a metabolic component underlying oxycodone tolerance, yet our results suggest the reversal of that tolerance by ethanol is not due to an alteration of the bio-disposition of oxycodone. We therefore conclude that ethanol reversal of oxycodone tolerance is mediated by specific neuronal mechanisms, and future experiments will be conducted to address this finding.

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Authorship Contributions:

The authors have no conflicts of interest to report.

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Footnotes

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Figure Legends

Figure 1. Ethanol reversal of oxycodone and hydrocodone tolerance. Ethanol fully reversed both oxycodone (A) and hydrocodone (B) tolerance at a dose of 1g/kg. Each data point is represented by a minimum of five mice and represented as mean ± SEM. Animals were injected once hourly with either saline or an ED_{80} dose of oxycodone or hydrocodone s.c. for six hours, followed by an i.p. injection of ethanol (1g/kg) or saline one hour later. 30 minutes later, various challenge doses of oxycodone or hydrocodone were injected s.c. to construct dose response curves and generate ED_{50} values.

Figure 2. **Intraperitoneal and oral ethanol reversed analgesic tolerance to s.c. oxycodone.** Mice were chronically injected with s.c. oxycodone (1.25 mg/kg) or saline hourly for 6 hours and treated with saline, i.p ethanol (1 g/kg) or po ethanol (2 g/kg) prior to receiving a challenge injection of 1.25 mg/kg oxycodone. Antinociception was assessed using the tail immersion assay where significant tolerance was displayed in mice chronically treated with oxycodone and no ethanol, compared to the acute oxycodone treatment group (* $P < 0.05$, One-way ANOVA). Two additional groups of mice were treated with repeated injections of oxycodone, but received either an ip injection of 1 g/kg ethanol or a gavage of 2 g/kg ethanol 30 minutes prior to receiving an oxycodone challenge injection. Ethanol treatment in these mice reversed tolerance development to oxycodone as seen by a restored response to the antinociceptive effects of oxycodone. Chronic oxycodone treated mice given either ip ethanol († $P < 0.01$) or oral ethanol (‡ $P < 0.05$) displayed significantly greater antinociceptive effects in response to a challenge injection of oxycodone compared to chronic oxycodone mice given saline prior to an oxycodone challenge (One-way ANOVA). All groups are represented by a minimum of 5 mice with data shown as mean ± SEM.

Figure 3. **Oral Oxycodone Time Course: Antinociception vs. Brain Concentration.** After a single administration of 16 mg/kg oxycodone po, brain oxycodone concentrations were plotted against oxycodone's antinociceptive effect in the warm water tail withdrawal assay at various time points ranging from 5 – 480 minutes. All data points represent mean ± SEM from a minimum of 5 mice. Brain oxycodone concentrations increased during the first 20 minutes where a plateau was observed until 30 minutes. Antinociception was slower to reach 100% MPE, which was not observed until 30 minutes and persisted until 60 minutes. Brain oxycodone concentrations at 60 minutes were much lower, measuring closer to the 5-minute time point, despite maximum antinociception. Significant observations for brain oxycodone concentrations were only noted at 120 (†$P < 0.05$) and 480 (‡$P < 0.001$) minute time points (One-way ANOVA), where concentrations were lower than all other time points. Antinociception was significantly higher at 20 (*$P < 0.05$), 30 and 60 minutes (***$P < 0.001$) compared to 5 minutes. Antinociception at 30 and 60 minutes was significantly higher compared to 240 ($P < 0.001$) and 480 minutes ($P < 0.0001$).

Figure 4. **Brain Oxycodone Concentrations Do Not Correlate with Antinociceptive Effects of Oxycodone.** Mice were injected or gavaged with the respective ED_{80} dose of oxycodone, 1.25 mg/kg s.c. and 16 mg/kg po. Both doses produced equal antinociception in mice, however, brain oxycodone concentrations significantly differed, with much higher concentrations detected after subcutaneous administration ($P < 0.0001$, Student's unpaired two-tailed t-test). A minimum of 5 mice were used for each dose tested, with bars representing means ± SEM.

Figure 5. **Acute and Chronic Oxycodone Brain Concentrations.** (A) Oxycodone brain concentrations 20 minutes following a challenge gavage of 16 mg/kg oxycodone in mice either

naïve to oxycodone or chronically treated with 64 mg/kg b.i.d. for four days. Acute concentrations represent the mean ± SEM of 10 mice, while chronic concentrations represent the mean ± SEM of 13 mice. Brain oxycodone concentrations detected 20 minutes following the oxycodone challenge were significantly lower in mice chronically treated with oxycodone compared to that of acutely treated mice (****$P < 0.001$, Student's two-tailed unpaired t-test). (B) The effects of 2g/kg ethanol were assessed against acute oxycodone brain levels. Each bar represents the mean ± SEM of at least 10 mice. Ethanol did not have had a significant effect ($P > 0.05$, Student's unpaired two-tailed t-test) and both groups displayed similar brain oxycodone levels. (C) The effects of 2g/kg ethanol were assessed against chronic oxycodone brain levels. Ethanol did not have a significant effect ($P > 0.05$, Student's unpaired two tailed t-test) and both groups displayed similar brain oxycodone levels in response to a challenge oxycodone gavage following chronic oxycodone treatment. Each bar represents the mean ± SEM of at least 8 mice.

TABLE 1

Comparison of Tolerance Development and Ethanol Reversal of Various Opioid Compounds

ED_{50}s and 95% confidence limits were calculated under acute, chronic and chronic + ethanol conditions in mice. All opioids tested produced antinociceptive tolerance when repeatedly administered subcutaneously. Oxycodone and hydrocodone were also evaluated for oral antinociceptive potencies and tolerance development. Only oral oxycodone produced significant tolerance to itself after repeated administration. * denotes a significant shift from acute ED_{50} values, determined by confidence limits that no longer overlap.

	Morphine s.c.	**Oxycodone s.c.**	**Oxycodone p.o.**	**Hydrocodone s.c.**	**Hydrocodone p.o.**
Acute ED_{50}	4.8 mg/kg (2.5 – 5.7)	0.89 mg/kg (0.72 – 1.12)	8.29mg/kg (6.12 – 11.52)	3.92 mg/kg (3.26 – 4.71)	38.79 mg/kg (29.21 – 51.53)
Chronic ED_{50}	19.9 mg/kg* (14.8 – 29.1)	1.70 mg/kg* (1.42 – 2.03)	33.41 mg/kg * (25.50 – 44.17)	9.01 mg/kg * (6.44 – 12.62)	55.92 mg/kg (40.63 – 76.96)
Chronic + Ethanol ED_{50}	5.2 mg/kg (4.9 – 5.5)	1.02 mg/kg (0.77 – 1.37)	35.52 mg/kg (20.19 – 59.03)	4.73 mg/kg (3.51 – 6.38)	N/A

Figure 1

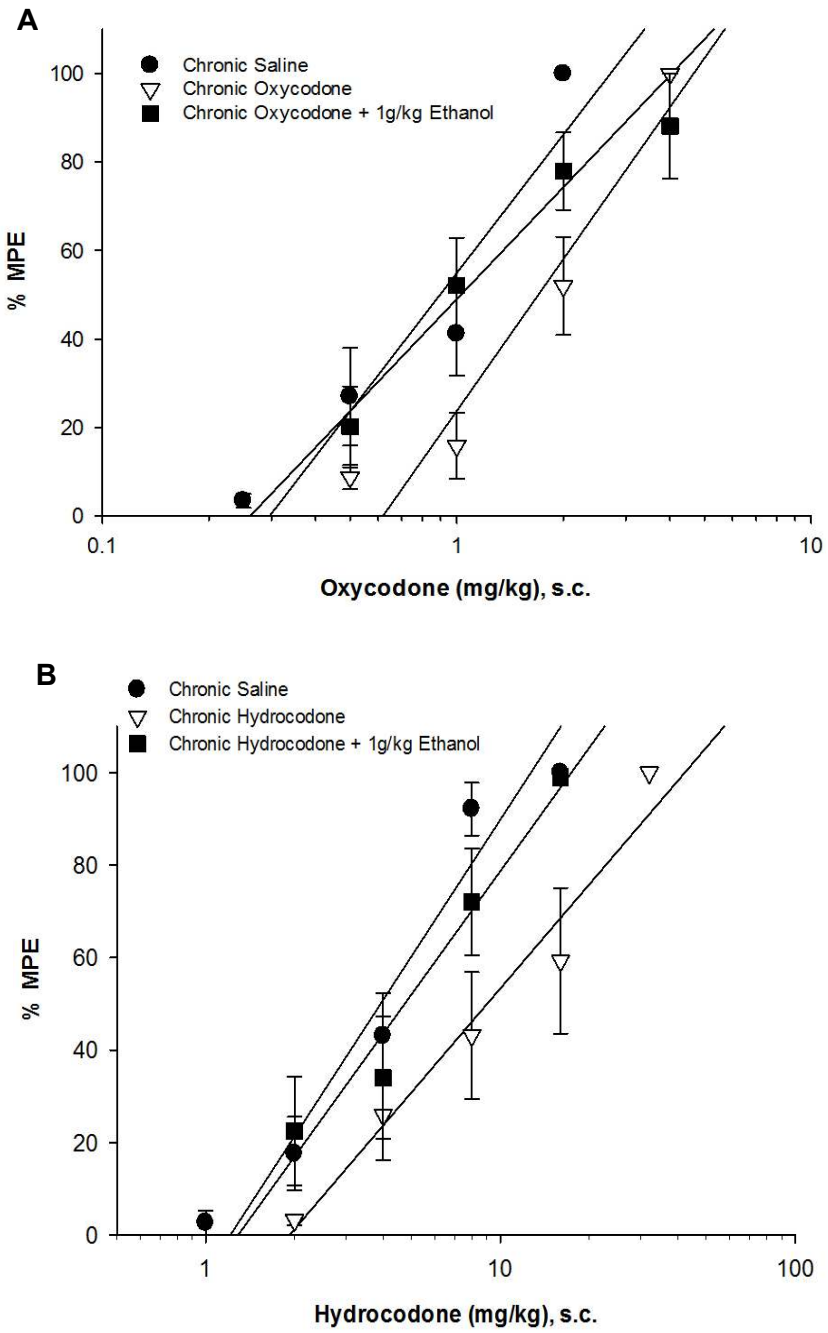

A
Chronic Saline
Chronic Oxycodone
Chronic Oxycodone + 1g/kg Ethanol
% MPE
0
20
40
60
80
100
0.1
1
10
Oxycodone (mg/kg), s.c.
B
Chronic Saline
Chronic Hydrocodone
Chronic Hydrocodone + 1g/kg Ethanol
% MPE
0
20
40
60
80
100
1
10
100
Hydrocodone (mg/kg), s.c.

Figure 2

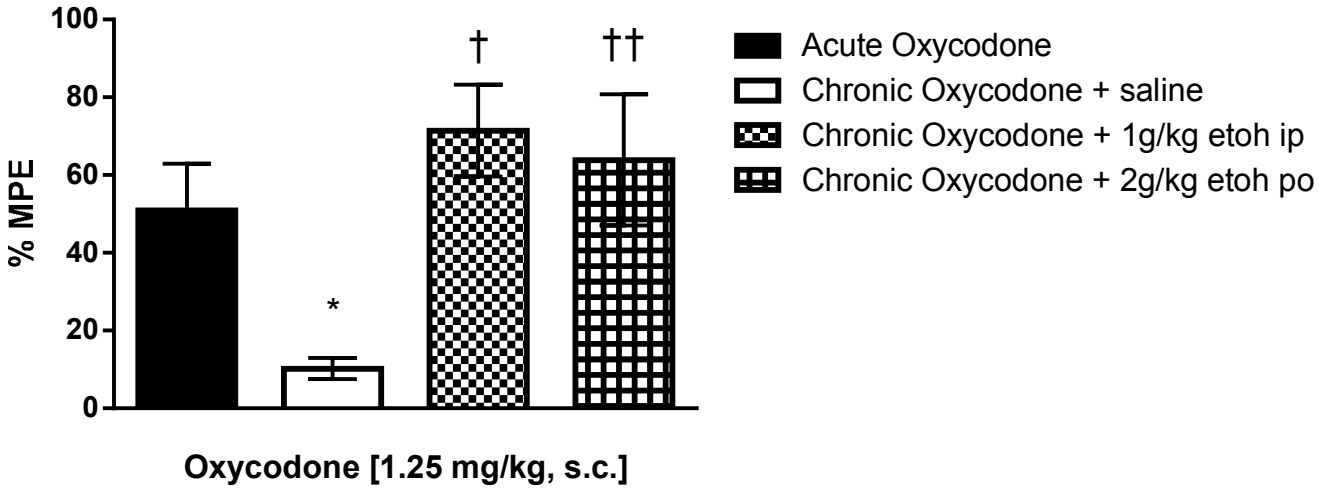

100
80
60
40
20
0
% MPE
*
†
††
Acute Oxycodone
Chronic Oxycodone + saline
Chronic Oxycodone + 1g/kg etoh ip
Chronic Oxycodone + 2g/kg etoh po
Oxycodone [1.25 mg/kg, s.c.]

Figure 3

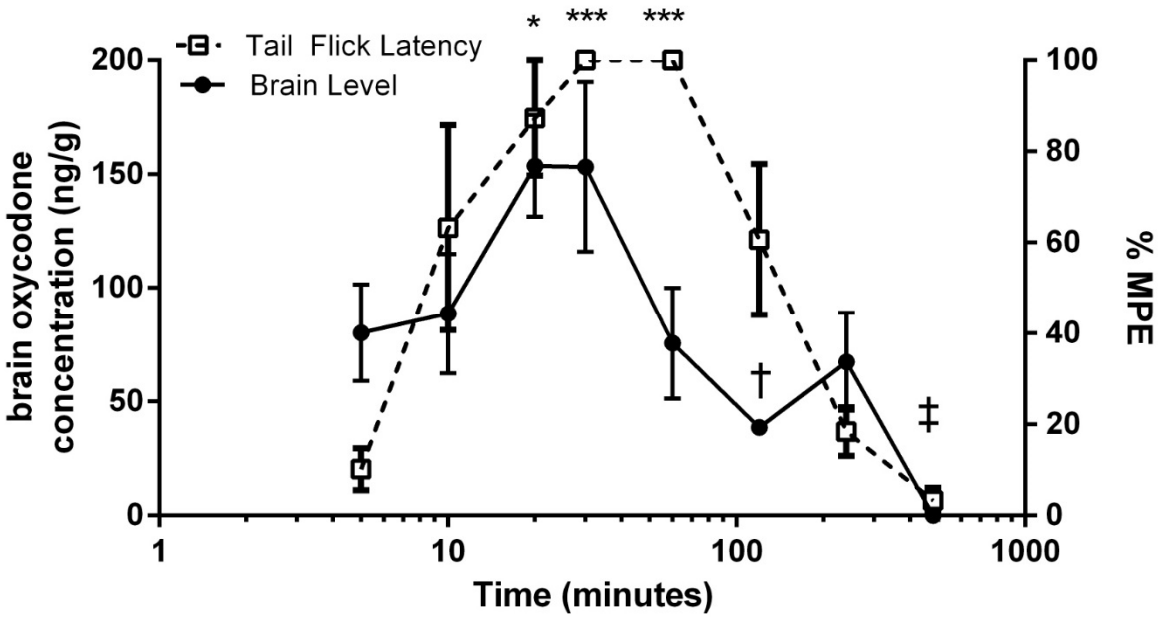

Tail Flick Latency
Brain Level
brain oxycodone concentration (ng/g)
% MPE
Time (minutes)
200
150
100
50
0
100
80
60
40
20
0
1
10
100
1000
*
***
***
†
‡

Figure 4

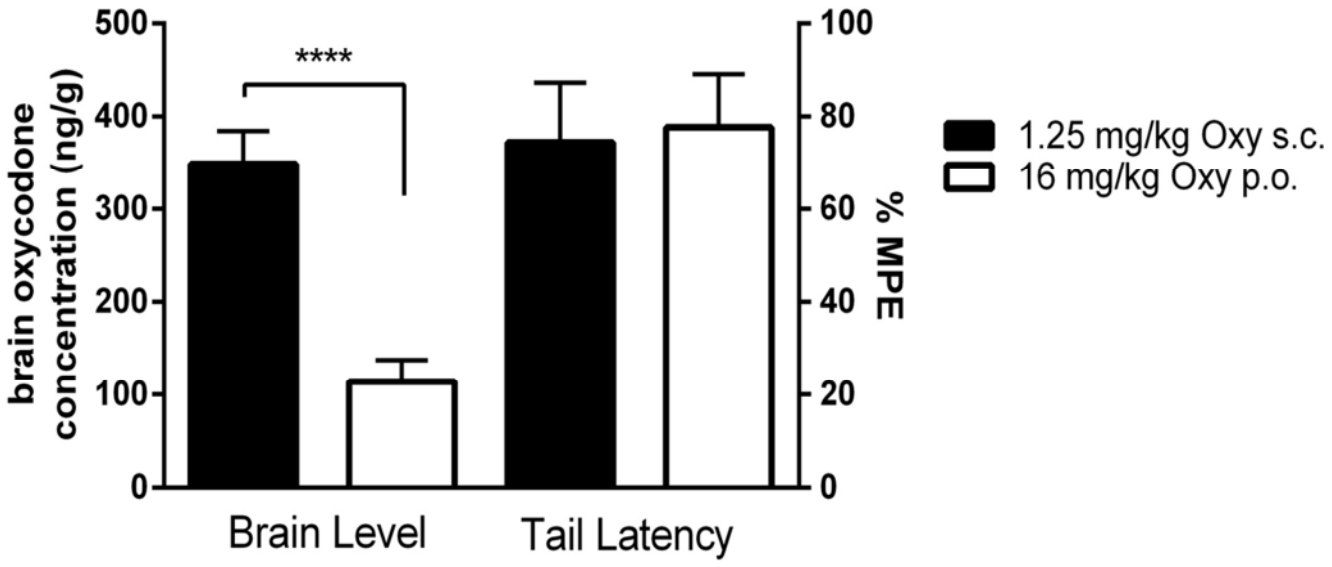
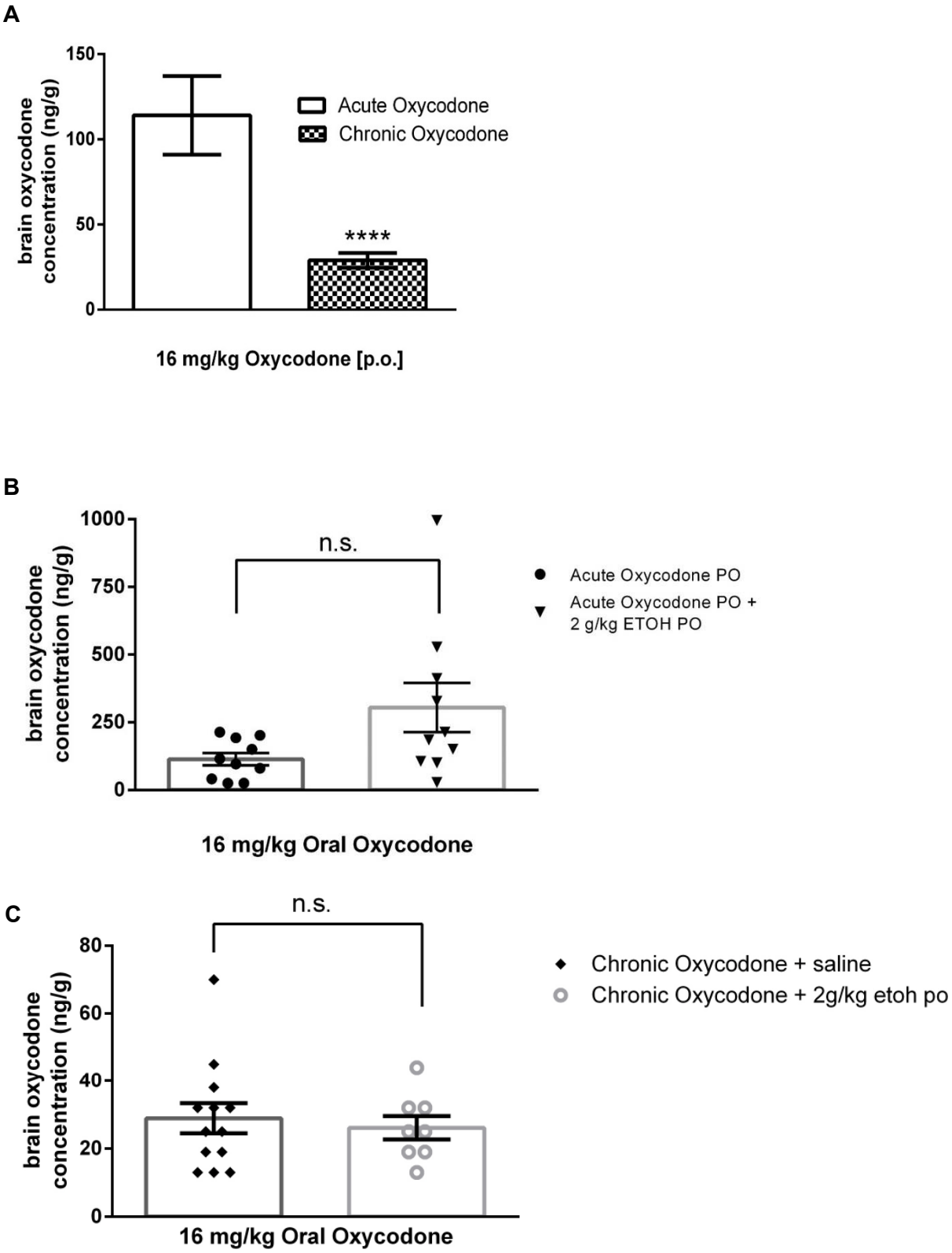

brain oxycodone concentration (ng/g)
500
400
300
200
100
0
****
100
80
60
40
20
0
% MPE
Brain Level
Tail Latency
1.25 mg/kg Oxy s.c.
16 mg/kg Oxy p.o.

Figure 5


A
150
100
50
0
brain oxycodone concentration (ng/g)
Acute Oxycodone
Chronic Oxycodone
****
16 mg/kg Oxycodone [p.o.]
B
1000
750
500
250
0
brain oxycodone concentration (ng/g)
n.s.
Acute Oxycodone PO
Acute Oxycodone PO + 2 g/kg ETOH PO
16 mg/kg Oral Oxycodone
C
n.s.
80
60
40
20
0
brain oxycodone concentration (ng/g)
Chronic Oxycodone + saline
Chronic Oxycodone + 2g/kg etoh po
16 mg/kg Oral Oxycodone