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Oxycodone-induced Tolerance to Respiratory Depression: Reversal by Ethanol, Pregabalin, and Protein Kinase C Inhibition.

Short Title: Ethanol and pregabalin reverse oxycodone tolerance

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Non standard abbreviations

AUC	area under the curve
DOPr	δ opioid receptor
KOPr	κ opioid receptor
MOPr	μ opioid receptor
NorBNI	Norbinaltorphimine

Key words

Opioid; oxycodone; respiratory depression; tolerance; protein kinase C

ABSTRACT

Background and Purpose:

Oxycodone, a prescription opioid, is a major drug of abuse, especially in the USA, and contributes significantly to opioid overdose deaths each year. Overdose deaths result primarily from respiratory depression. We have studied respiratory depression by oxycodone and have characterized how tolerance develops on prolonged exposure to the drug. We have investigated the role of protein kinase C (PKC) in maintaining tolerance and have examined whether ethanol or pregabalin reverse oxycodone-induced tolerance

Experimental Approach:

Respiration was measured in male CD-1 mice by whole body plethysmography. Mice were preinjected with oxycodone, then implanted with mini-pumps (s.c.) delivering 20, 45 or 120 $mg.kg^{-1}.d^{-1}$ oxycodone for 6 d and subsequently challenged with oxycodone (3 $mg.kg^{-1}$ i.p.) or morphine (10 $mg.kg^{-1}$ i.p.) to assess the level of tolerance.

Key Results:

Oxycodone-treated mice developed tolerance to oxycodone and cross tolerance to morphine-induced respiratory depression. Tolerance was less with 20 $mg.kg^{-1}.d^{-1}$ than with 45 or 120 $mg.kg^{-1}.d^{-1}$ oxycodone treatment. At doses that do not depress respiration, ethanol (0.3 $g.kg^{-1}$), pregabalin (20 $mg.kg^{-1}$) and calphostin C (45 $\mu g.kg^{-1}$) all reversed oxycodone-induced tolerance resulting in significant respiratory depression. Reversal of tolerance was less in mice treated with oxycodone (120 $mg.kg^{-1}.d^{-1}$). In mice receiving ethanol and calphostin C or ethanol and pregabalin there was no greater reversal of tolerance than seen with either drug alone.

Conclusion and Implications:

These data suggest that oxycodone-induced tolerance is mediated by PKC and that reversal of tolerance by ethanol or pregabalin may be a contributory factor in oxycodone overdose deaths.

INTRODUCTION

Opioid overdose deaths are a prominent public health issue in many countries around the world. In the USA deaths involving prescription opioids and illicit heroin increased 5 fold from 1999 to 2015 (Hedegaard et al, 2017) despite considerable efforts aimed at combating the problem (Okie, 2010). In 2015 the number of overdose deaths involving prescription opioids in the USA (~ 17,000) was greater than that from illicit heroin (~13,000). Death due to opioid overdose results primarily from respiratory depression (White & Irvine, 1999). Oxycodone (Oxycontin) is one of the most commonly prescribed opioid analgesics in the USA and forms a significant percentage of the hospital admissions for opioid overdose (Pfister *et al.*, 2016) and prescription opioid overdose deaths (Inciardi *et al.*, 2007, Kenan *et al.*, 2012, Sgarlato and deRoux, 2015). In most instances of prescription opioid overdose other drugs such as alcohol and benzodiazepines were also found to be present (Sgarlato & deRoux, 2015). We and others have suggested that polydrug use (i.e. using other drugs such as alcohol, benzodiazepines, or pregabalin in addition to opioids) may increase the likelihood of opioid overdose (Darke & Hall, 1995, 2003; Hickman *et al*, 2007; Brecht *et al*, 2008; Hill *et al*, 2016; Lyndon *et al*, 2017).

We have recently reported that in mice, tolerance develops to the respiratory depressant effects of morphine, an active metabolite of heroin (Hill *et al*, 2016). The tolerance induced by morphine was reversed by a single low dose of ethanol that itself did not induce respiratory depression, thus resulting in significant respiratory depression to a dose of morphine in otherwise morphine tolerant animals. In contrast, the tolerance induced by prolonged treatment with methadone was not reversed by ethanol. This may be explained by morphine and methadone inducing μ-opioid receptor (MOPr) desensitization and tolerance by different cellular mechanisms (for review see Williams *et al*, 2013). There is substantial evidence that

MOPr desensitization and tolerance to morphine is mediated primarily by a protein kinase C (PKC)-dependent mechanism (Bailey *et al*, 2006, 2009a, 2009b) whereas desensitization and tolerance to methadone is likely mediated by a G protein-coupled receptor kinase (GRK)- and arrestin-dependent mechanism (McPherson *et al*, 2010). We have recently gone on to show that tolerance to the respiratory-depressant effects of morphine can also be reversed by acute injection of the gabapentoid drug pregabalin (Lyndon *et al.*, 2017).

Oxycodone depresses respiration in man (Leino et al., 1999; Chang et al., 2010) and in rodents (Kuo et al., 2015; Whiteside et al., 2016). In the present experiments we have characterized the respiratory depressant effects of oxycodone in mice. We have examined whether prolonged oxycodone administration results in the development of tolerance to respiratory depression, the role of PKC in such tolerance and whether acute administration of a low dose of ethanol or pregabalin, drugs that may be taken by people also using oxycodone, could reverse oxycodone-induced tolerance.

METHODS AND MATERIALS

Animals: Male CD-1 mice (Harlan Laboratories, UK) weighing approximately 30 g were group housed, 4 - 6 per cage and maintained at 22 °C on a reversed 12 h dark-light cycle with food and water available *ad libitum*. Experiments were performed in the dark (active) phase. A total of 291 mice were used in the study. All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, the European Communities Council Directive (2010/63/EU) and the University of Bristol ethical review document, as well as the ARRIVE and British Journal of Pharmacology guidelines.

Measurement of respiration: Respiration was measured in freely moving animals using plethysmography chambers (EMKA Technologies, France) supplied with a 5% CO_2 in air mixture (BOC Gas Supplies, UK). For a detailed description of measuring respiratory function by whole body plethysmography see Lim et al. (2014). Animals were randomly ascribed to treatment groups with the experimenter blinded to drug treatment until after subsequent data analyses had been performed. Rate and depth of respiration were recorded and averaged over 5 min periods (except immediately after drug injection when the time-period was 3 min) and converted to minute volume (rate x tidal volume). Data are presented both as minute volume and as percentage change from the pre-drug minute volume baseline, calculated for each mouse individually before mean data were plotted. Presenting data as percentage change from the pre-drug levels has been performed to control for variation between treatment groups that may have different baseline levels of respiration. In our experience variation in baseline respiration levels do not influence the extent of opioid depression of respiration (Hill & Henderson, 2017, unpublished data).

Data from previous experiments where respiratory depression was measured either following acute opioid administration in naïve mice or following pump implantation were subjected to post hoc power analyses using G*Power (version 3.1.9). Our calculations indicated that n=6 (acute experiments) or n=7 (pump experiments) for each individual group would produce a significant result if an actual effect occurred.

The acute effects of opioid agonists (oxycodone, morphine and U69593) on respiration (minute volume) were monitored for 30 min following drug administration. The opioid antagonists naloxone, naltrindole and norbinaltorphimine (NorBNI) were administered 30 min prior to the opioid agonists.

Induction of opioid tolerance: We have previously reported that in the mouse tolerance to the respiratory depressant effects of morphine did not develop with repeated twice daily doses of morphine (Hill et al., 2016) but did develop with continuous administration of morphine from either a morphine pellet or a subcutaneously implanted osmotic mini-pump (Withey et al., 2017). Oxycodone is metabolized more rapidly in rodents than in man (Raehal and Bohn, 2011). In the present experiments oxycodone tolerance was induced by priming mice with three i.p. injections of oxycodone at 12 h intervals followed by subcutaneous implantation on the dorsal flank of an osmotic mini-pump (ALZET®) containing oxycodone for 6 days. This follows the protocol first described for induction of opioid tolerance in mice by Quillinan et al., (2011). Implantation of osmotic mini-pumps was done under isoflurane general anaesthesia. Three schedules of oxycodone treatment were used:-

(i) Low dose treatment – 3 x 30 $mg.kg^{-1}$ i.p injections at 12 h intervals followed by 6 days of 20 $mg.kg^{-1}.d^{-1}$ s.c. The oxycodone dosing is based on the relative potency

of oxycodone and morphine to depress respiration in mice (see Fig. 1) and the protocol described by Withey et al., (2017) for induction of morphine tolerance.

(ii) Moderate dose treatment – 3 x 100 $mg.kg^{-1}$ i.p injections at 12 h intervals followed by 6 days of 45 $mg.kg^{-1}.d^{-1}$ s.c.. In this treatment, the doses of oxycodone are the same as the doses morphine used by Withey et al., (2017).

(iii) High dose treatment – 3 x 100 $mg.kg^{-1}$ i.p injections at 12 h intervals followed by 6 days of 120 $mg.kg^{-1}.d^{-1}$ s.c. In this treatment, the daily dose of oxycodone released from the pump has been increased six-fold from the low dose treatment.

Assessment of opioid tolerance: To assess the level of tolerance induced by the oxycodone treatments, mice were injected on day 6 following oxycodone pump implantation with a challenge dose of morphine (10 $mg.kg^{-1}$ i.p.) or oxycodone (3 $mg.kg^{-1}$ i.p.) and respiration monitored for 30 min. The degree of respiratory depression observed in opioid-treated animals was compared to that observed in control animals that had received saline priming injections and been implanted with saline filled osmotic mini-pumps rather than opioid drug and which had been challenged with morphine (10 $mg.kg^{-1}$ i.p.). We used the same morphine challenge to assess oxycodone-induced tolerance as we have used in previous studies of tolerance induced by prolonged morphine and methadone treatments (Hill et al., 2016; Withey et al., 2017; Lyndon et al., 2017) in order that the levels of tolerance induced by different opioids can be directly compared. In addition, we have also used an oxycodone challenge to determine the degree of tolerance that prolonged oxycodone treatment induces to oxycodone itself.

Reversal of tolerance: The ability of ethanol (0.3 $g.kg^{-1}$ i.p.), pregabalin (20 $mg.kg^{-1}$ i.p.) and calphostin C (45 $\mu g.kg^{-1}$ i.p.) to reverse tolerance to respiratory depression induced by

prolonged oxycodone treatment was investigated. Ethanol and pregabalin were administered simultaneously with the acute morphine challenge whereas calphostin C was administered as a pre-treatment 30 min before the acute morphine challenge. In control experiments the appropriate vehicle was injected with the morphine challenge.

Measurement of Brain and Plasma Oxycodone Levels: Mice were killed using escalating CO_2 and blood samples collected from the descending abdominal aorta. Blood samples were centrifuged at 3000 *g* for 10 min at 4°C and the aliquoted plasma supernatant stored at −20°C. Immediately after blood sampling, mice were decapitated and the head placed on ice. After removal from the skull, the brains were flash frozen in liquid nitrogen before storage at −80°C. Subsequently plasma and brain levels of oxycodone were measured by gas chromatography mass spectrometry analysis at the Virginia Commonwealth University (Richmond, VA, USA) by the method described in Jacob *et al*. (2017).

Data Analysis: Area under the curve (AUC) was determined using a 100% baseline as described previously (Hill *et al.,* 2016). Overall changes from a single factor were analyzed using a One-way ANOVA with Bonferroni's post-test. Interaction between prolonged drug treatment and challenge drug was analysed using a Two-way ANOVA in a two-by-two factorial. Changes in groups over time with repeat measurements were analysed using a Two-way repeated measures ANOVA with Bonferroni's post-test to analyse drug effect over time. GraphPad Prism 5 was used for all statistical analyses. All data are displayed as mean ± standard error of the mean (SEM). The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al. 2015).

Drugs and chemicals

Morphine hydrochloride (Macfarlane Smith), naloxone hydrochloride, naltrindole hydrochloride, norbinaltorphimine dihydrochloride, oxycodone hydrochloride and (+)-(5α,7α,8β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U69593) (all from Sigma Aldrich, UK) as well as S-pregabalin (extracted and purified from Lyrica tablets by Dr Erica Burnell, University of Bristol) were dissolved in sterile saline. Ethanol (Sigma Aldrich, UK) was diluted in sterile saline. Calphostin C (Tocris, UK) was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in distilled water such that the final concentration of DMSO was 0.5%.

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a, 2017b).

RESULTS

Comparison of the respiratory depressant effects of oxycodone and morphine

We have previously demonstrated the respiratory depressant effect of morphine (3 and 10 $mg.kg^{-1}$ i.p.) in mice breathing 5% CO_2 in air (Hill *et al.,* 2016). Acute administration of oxycodone (1 or 3 $mg.kg^{-1}$ i.p.) produced dose-dependent depression of respiration (Fig. 1A, B, E, G). Oxycodone was slightly more potent in depressing respiration than morphine, in that 3 $mg.kg^{-1}$ oxycodone produced a similar degree of respiratory depression as that to 10 $mg.kg^{-1}$ morphine (Fig. 1G). No mice were insensitive to the respiratory depressant effects of oxycodone at the doses tested (Fig. 1G). The depression of respiration by oxycodone resulted from a decrease in the rate of respiration (control rate 550 ± 33 breaths per min versus 291 ± 22 breaths per min at the peak of inhibition by oxycodone 3 $mg.kg^{-1}$) (Student's unpaired T-test, t=6.50, dfn=10, P<0.05), rather than a decrease in tidal volume (control tidal volume 0.29 ± 0.02 $ml.min^{-1}$ versus 0.28 ± 0.01 $ml.min^{-1}$ at the peak of inhibition by oxycodone $mg.kg^{-1}$) (Student's unpaired T-test, t=0.45, dfn=10, P<0.05). As previously reported for morphine (Hill *et al.*, 2016), although tidal volume was maintained in the presence of oxycodone the duration of inspiration was prolonged whilst the depth of respiration was reduced (Fig. 1E, F & H). Tidal volume was maintained because the duration of inspiration was prolonged by an apneustic compensation (see also Hill *et al*., 2016). Mice did not appear to exhibit ribcage muscle stiffness during oxycodone treatment.

Prior administration of the opioid receptor antagonist naloxone (1 $mg.kg^{-1}$ i.p., 30 mins prior to opioid agonist) inhibited the respiratory depressant effects of both oxycodone (3 $mg.kg^{-1}$) and morphine (10 $mg.kg^{-1}$) whereas the selective δ-opioid receptor (DOPr) antagonist

naltrindole (20 mg.kg^{-1} i.p., 30 mins prior to opioid agonist) had no effect on the respiratory depressant effect of either opioid (Fig. 2A and B). Treatment with the κ-opioid receptor (KOPr) antagonist NorBNI (10 mg.kg^{-1} i.p.) 24 h prior to the opioid administration did not affect the respiratory depressant effect of oxycodone (3 mg.kg^{-1}) or morphine (10 mg.kg^{-1})(Fig. 2A and B) but did however prevent the antinociceptive response to the KOPr agonist U69593 (20 mg.kg^{-1} i.p.) in the tail flick latency assay (latencies to withdrawal - control 3.6 ± 0.9 s; U69593 6.1 ± 0.5 s; NorBNI + U69593 3.3 ± 0.5 s (N = 6 in all groups) (U69593 vs control or NorBNI + U69593 $P<0.05$, One-way ANOVA with Bonferroni's post-test, $F=5.42$, dfn=2, dfd=18) proving that the antagonist was active in these *in vivo* experiments.

Development of tolerance to oxycodone and cross-tolerance to morphine following prolonged oxycodone administration.

To induce tolerance with oxycodone we used a similar schedule of priming doses followed by pump implantation to that we have used previously for morphine and methadone (Withey *et al.*, 2016). We examined three oxycodone treatment schedules - low dose, moderate dose and high dose (for details of the dosage regimes see Methods and Materials). The plasma and brain levels of oxycodone measured on day 6 of treatment are given in Table 1. By day 6 the respiratory rate of mice in each oxycodone treatment group was not different from that of saline-treated control mice (minute volume values (ml.min^{-1}) 184 ± 12 saline-treated; 174 ± 17 low dose oxycodone; 175 ± 10 medium dose oxycodone; 186 ± 13 high dose oxycodone) (One-way ANOVA with Bonferroni's post-test, $F=0.214$, dfn=3, dfd=28, $P>0.05$). Without removing the oxycodone-containing pump mice were then challenged with oxycodone (3 mg.kg^{-1} i.p.) or morphine (10 mg.kg^{-1} i.p.) to assess the degree of tolerance that had been induced. Prolonged medium dose oxycodone treatment reduced the response to acute

oxycodone challenge by 68% (Fig. 3E & F). Prolonged low dose oxycodone treatment reduced the response to acute morphine challenge by 42% whereas prolonged medium and high dose oxycodone treatment decreased the response to acute morphine challenge by over 88% and 80% respectively (Fig. 3 A-D). The reductions in the morphine challenge responses induced by the prolonged medium and high dose oxycodone treatments were not statistically different from each other.

Ethanol and pregabalin reversal of tolerance induced by oxycodone

We have previously reported that a single, low dose of ethanol reversed morphine-induced tolerance to respiratory depression in mice but did not reverse either the tolerance induced by prolonged methadone treatment or the blockade of morphine-induced respiratory depression produced by prolonged treatment with buprenorphine (Hill *et al.,* 2016). Similarly, we have shown that a single dose of S-pregabalin reversed morphine-induced tolerance to respiratory depression in mice (Lyndon *et al.*, 2017). We therefore examined the ability of ethanol and pregabalin to reverse tolerance induced by prolonged oxycodone treatment. To do this we used doses of ethanol (0.3 $g.kg^{-1}$ i.p.) and pregabalin (20 $mg.kg^{-1}$ i.p.) that do not depress respiration (Hill *et al.*, 2016; Lyndon *et al.*, 2017).

After prolonged treatment with medium or high dose oxycodone, mice that received ethanol (0.3 $g.kg^{-1}$ i.p.) simultaneously with the challenge dose of morphine (10 $mg.kg^{-1}$) showed significantly greater respiratory depression compared to mice that received only morphine challenge following prolonged oxycodone treatment (Fig. 4A). indicating that ethanol had reversed the oxycodone-induced tolerance. This low dose of ethanol alone did not cause significant respiratory depression in medium or high dose oxycodone pump-implanted mice

(Fig. 4). Tolerance induced by the medium dose oxycodone treatment was reversed by ethanol to 84% of control. Tolerance induced by high dose oxycodone treatment was reversed to 55% of control.

In mice that had received the medium dose oxycodone treatment acute administration of pregabalin (20 $mg.kg^{-1}$ i.p.) partially restored the respiratory depressant effect of the challenge dose of morphine (to 55% of control)(Fig. 4A). Pregabalin alone did not cause significant respiratory depression in medium or high dose oxycodone pump-implanted mice (Fig. 4). In mice that had received the high dose oxycodone treatment the restoration of the respiratory depressant effect of morphine, when pregabalin was co-administered, did not reach statistical significance (Fig. 4B).

We next examined whether ethanol and pregabalin would act additively to reverse tolerance induced by high dose oxycodone treatment. Oxycodone treated mice received an acute injection of pregabalin (20 $mg.kg^{-1}$ i.p) and ethanol (0.3 $g.kg^{-1}$ i.p.) along with morphine (10 $mg.kg^{-1}$ i.p.). With the combination of ethanol and pregabalin there was no greater reversal of oxycodone-induced tolerance than by ethanol alone (Fig. 4B).

Reversal of oxycodone-induced tolerance by calphostin C and lack of additivity with ethanol

There is substantial evidence for the role of PKC in desensitization of MOPrs by morphine and subsequent development of cellular tolerance on prolonged exposure to morphine (Bailey et al 2004; 2009a; 2009b; Levitt & Williams, 2012; Williams *et al.,* 2013). Similarly, PKC has been shown to be involved in the development of tolerance to the antinociceptive and respiratory depressant effects of morphine (Smith *et al.*, 2007; Withey *et al.*, 2017). We therefore sought

to investigate the role of PKC in tolerance induced by oxycodone using calphostin C, a brain-penetrant drug that inhibits both conventional and novel isoforms of PKC (Kobayashi *et al.*, 1989).

Calphostin C (45 μg.kg^{-1} i.p.) by itself had no direct effect on respiration in näive mice nor did it alter the acute respiratory depressant effect of morphine (10 mg.kg^{-1}) (Withey *et al.,* 2017). However, in mice that had received the chronic medium dose oxycodone treatment an acute injection of calphostin C (45 μg.kg^{-1} i.p.) 30 min prior to an acute morphine challenge completely restored the respiratory depressant effect of the morphine challenge (Fig. 4A). This effect was not observed in oxycodone treated mice injected with the vehicle for calphostin (0.5% DMSO in saline) prior to the morphine challenge (data not shown). As with ethanol and pregabalin, calphostin C alone did not cause significant respiratory depression in medium dose oxycodone pump-implanted mice (Fig. 4). These data are consistent with calphostin C reversing oxycodone-induced tolerance to respiratory depression. In mice that had received the high dose oxycodone treatment the acute injection of calphostin C (45 μg.kg^{-1} i.p.) resulted in partial reversal of oxycodone-induced tolerance (to 55% of control)(Fig. 4B).

We next examined whether ethanol and calphostin C would act additively to reverse tolerance induced by high dose oxycodone treatment. Oxycodone treated mice received an acute injection of calphostin C (45 μg.kg^{-1} i.p) 30 min prior to administration of ethanol (0.3 g.kg^{-1} i.p.) and morphine (10 mg.kg^{-1} i.p.). With the combination of ethanol and calphostin C there was no greater reversal of oxycodone-induced tolerance than by either drug alone (Fig. 4B).

DISCUSSION

The results of this study demonstrate that oxycodone, a prescription opioid and major drug of abuse, induces marked respiratory depression; that with prolonged oxycodone administration tolerance develops to this effect; and most interestingly that this tolerance is reversed by inhibition of PKC, by low dose ethanol, and by pregabalin. Thus, this study highlights the potential dangers of polydrug abuse when taking oxycodone either as a prescribed drug or as a drug of abuse.

We observed that in the mouse oxycodone was more potent than morphine in depressing respiration. The depression of respiration by oxycodone was reversed by naloxone, but not significantly altered by the DOPr antagonist naltrindole nor by the KOPr antagonist NorBNI indicating that the effect was mediated through activation of MOPr. Yang *et al.*, (2016) had reported that in MOPr knockout mice high doses of oxycodone (>40 $mg.kg^{-1}$ s.c.) induced naltrindole-reversible antinociception.

We have previously observed that tolerance to the respiratory depressant effects of morphine does develop, but does so more slowly than tolerance to antinociception (Hill *et al.*, 2016). The present study demonstrates that for respiratory depression prolonged (6 day) exposure to oxycodone also results in tolerance to oxycodone as well as cross tolerance to morphine. The degree of cross tolerance to morphine observed increased as the oxycodone treatment was increased. Oxycodone is eliminated in the rodent more rapidly than morphine (Raehal and Bohn, 2011) which may explain why we needed to use higher doses of oxycodone than morphine to induce equivalent levels of tolerance (compare the levels of tolerance induced by oxycodone in this paper with those observed for morphine in Hill *et al.*, 2016).

The tolerance to respiratory depression induced by oxycodone was reversed by calphostin C, a brain penetrant inhibitor of PKC (Kobayashi *et al.*, 1989). The fact that calphostin C reversed oxycodone-induced tolerance after 6 days of oxycodone treatment, indicates that ongoing PKC activity is required to maintain oxycodone tolerance. We have previously reported that calphostin C reversed morphine- but not methadone-induced tolerance (Withey *et al.*, 2017). Morphine and oxycodone have similar lower intrinsic agonist efficacy at MOPr for G protein activation whereas methadone has higher intrinsic efficacy (McPherson *et al.*, 2011). The agonist efficacy of oxycodone and morphine is still sufficient, however, for them to induce both analgesia and respiratory depression in man. It appears likely therefore that PKC is involved in tolerance to lower efficacy MOPr agonists but not to higher efficacy agonists. Multiple isoforms of PKC (PKC-α, -γ and –ε) have been identified as mediators of tolerance to the antinociceptive actions of morphine (Smith *et al.*, 2007). Of related interest, expression of constitutively active PKC*α* or PKC*ε* isoforms in the pre-Bötzinger complex, a group of neurons involved in the generation of respiratory rhythm that are inhibited by MOPr agonists, increased the development of tolerance to respiratory depression induced by morphine, an effect that afforded increased protection from death by overdose (Lin *et al.*, 2012).

The potential for other non-opioid drugs to contribute to overdose deaths involving opioids has long been recognized (see Hickman *et al.*, 2007). In the present study, we focused on two drugs, ethanol and pregabalin. Ethanol is frequently detected at post mortem along with oxycodone and other prescription opioids (Cone *et al.*, 2004; Häkkinen et al., 2012). Pregabalin is also used in the treatment of severe pain states, sometimes in combination with opioids. It has recently become a drug of abuse popular with heroin users and increasingly associated with opioid overdose deaths (Häkkinen *et al.*, 2014; Lyndon *et al.*, 2017). In

England and Wales there has been a dramatic increase in acute drug deaths involving pregabalin and its congener, gabapentin, in recent years (Lyndon et al., 2017). Data for 2016 reveal that in over 90% of deaths in which pregabalin or gabapentin was present at post mortem, an opioid was also present (Office for National Statistics, 2017).

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). In mice, low doses of ethanol have previously been shown to reverse morphine-induced cellular tolerance (Llorente *et al.*, 2013), antinociception tolerance (Hull *et al.*, 2013) and tolerance to respiratory depression (Hill *et al.*, 2016). Ethanol also reverses oxycodone-induced antinociception tolerance (Jacob *et al.*, 2017) as well as tolerance to respiratory depression as shown in this paper.

Acute ethanol administration in mice at doses that reversed tolerance did not alter brain concentrations of either oxycodone (Jacob *et al.*, 2017) or morphine (Hill *et al.*, 2016) suggesting that ethanol reversal of tolerance to these opioids is not due to altered drug distribution or other pharmacokinetic changes. Reversal of oxycodone-induced tolerance by ethanol did not summate with the reversal induced by calphostin C. This may indicate that these two drugs act to reduce PKC activity. Attempts to demonstrate ethanol inhibition of PKC have produced mixed results. Reneau *et al.*, (2011) demonstrated that ethanol (1 – 100 mM) inhibited PKCα activity *in vitro* by up to 40%, although we and others found less inhibition of PKCα activity by ethanol (Slater *et al.*, 1997; Rex *et al.*, 2008; Llorente *et al.*, 2013). However, such *in vitro* studies using purified PKC are likely to be hindered by the absence of cofactors that are required for ethanol inhibition of the enzyme and also do not examine the initial translocation of PKC from the cytoplasm to the plasma membrane.

There have been several recent reports that gabapentin and pregabalin, are being misused with subsequent development of dependence (Mersfelder and Nichols, 2016, Papazisis and Tzachanis, 2014, Schjerning *et al.*, 2016, Smith *et al.*, 2016). Additionally, pregabalin misuse is increasingly observed to be associated with opioid use, in particular, heroin users report that pregabalin reinforces or enhances the rewarding effects of heroin (Bastiaens *et al.*, 2016, Evoy *et al.*, 2017, Grosshans *et al.*, 2013, McNamara *et al.*, 2015, Smith *et al.*, 2015, Lyndon *et al.*, 2017). We have previously demonstrated that in mice, a dose of pregabalin that did not in itself depress respiration, reversed tolerance to respiratory depression induced by prolonged morphine treatment, thus increasing opioid-induced respiratory depression (Lyndon *et al.,* 2017). At higher doses pregabalin alone depressed respiration an effect that summated with that of morphine. In the present study, we have shown that pregabalin partially reversed tolerance to respiratory depression induced by oxycodone. We are not aware of any evidence to date that pregabalin or gabapentin directly inhibit PKC, but gabapentin has been shown to reduce PKC translocation to the plasma membrane in rat spinal cord (Zhang et al., 2015). However, the mechanism by which pregabalin reverses oxycodone tolerance remains to be elucidated. Our observation that pregabalin reverses oxycodone tolerance highlights the potential risk of concomitant use of pregabalin and oxycodone either in patients taking these drugs to treat pain states or in people misusing these drugs.

CONCLUSIONS

Our data provide compelling evidence for an interaction between ethanol or pregabalin and oxycodone to reverse tolerance to the respiratory depressant effects of oxycodone. We conclude from this study that ethanol or pregabalin reversal of tolerance to the respiratory

depression induced by oxycodone may be an important contributing factor in deaths involving this drug.

AUTHOR CONTRIBUTIONS

RH, WLD, EK and GH participated in research design

RH performed the experiments

RH and GH analysed the data

RH, WLD, EK and GH participated in writing the manuscript.

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Table 1. Plasma and brain levels of oxycodone following prolonged (6 d) treatments.

Oxycodone treatment	**Low**	**Medium**	**High**
Plasma concentration ($ng.mL^{-1}$)	81 ± 8	284 ± 147	1670 ± 635
Brain level ($ng.g^{-1}$)	248 ± 126	426 ± 87	1703 ± 665

N = 6 in all cases except the plasma concentration following medium oxycodone treatment where N = 5 (one sample lost)

Figure legends

Figure 1. Depression of respiration by oxycodone and morphine. Oxycodone (1 and 3 mg.kg^{-1}, i.p.) and morphine (1 – 10 mg.kg^{-1}, i.p.) dose-dependently depressed mouse respiration. Saline did not depress respiration. In **A** and **C** data are presented as minute volume whereas in **B** and **D** the level of respiratory depression seen following drug injection is expressed as a percentage change in the pre-drug minute volume baseline, calculated for each mouse individually before mean data were plotted. In **E** and **F** the effect of oxycodone on respiratory rate and tidal volume are shown. Data are from the same groups of mice as in **A** and **B**. In **G** the data in **B** and **D** have been recalculated and plotted as the area under curve (AUC). The AUC for the percentage change in minute volume has been calculated for each individual animal before the mean AUC has been calculated. The data were analyzed using a One-way ANOVA with Bonferroni's comparison ($F=12.6$, dfn=4, dfd=30, $P<0.05$, N = 6 for all groups). In **A** - **G** data are expressed as mean ± SEM. In **H** are shown raw respiration traces recorded from a single mouse before and after administration of oxycodone (3 mg.kg^{-1}, i.p.). The thin horizontal line indicates the point of pressure inflexion. On the respiration traces inspiration is downwards. *$p<0.05$, ns non significant; N=6 for all groups.

Figure 2. Effect of opioid receptor antagonists on the depression of respiration by oxycodone and morphine. Naloxone (NLX; 1 mg.kg^{-1}, i.p.), administered 30 min prior, markedly reduced the respiratory depression induced by oxycodone (3 mg.kg^{-1}, i.p.) ($F=18.82$, dfn=3, dfd=24, $P<0.05$) and abolished that induced by morphine (10 mg.kg^{-1}, i.p.) ($F=34.57$, dfn=3, dfd=24, $P<0.05$). Naltrindole (NLT; 20 mg.kg^{-1} i.p.), administered 30 min prior, had no effect on the respiratory depression induced by oxycodone or morphine. Treatment with NorBNI (10 mg.kg^{-1}, i.p.) 24 h prior to opioid administration did not affect respiratory depression induced by oxycodone or morphine. Data are expressed as mean ± SEM and were

compared using a one-way ANOVA with Bonferroni's comparison. *p<0.05, ns non significant; N=6 for all groups.

Figure 3. Tolerance to respiratory depression induced by prolonged oxycodone treatment. **A**, **B** and **C** show the depression of respiration induced by a challenge dose of morphine (10 $mg.kg^{-1}$ i.p.) in mice that had received prolonged low, medium or high dose oxycodone treatment. In **D** the area under the curve (AUC) for the percentage change in minute volume in **A - C** has been determined for each individual animal before the mean AUC has been calculated and compared to that observed in animals that had not received prolonged oxycodone treatment. The depression of respiration induced by the challenge dose of morphine (10 $mg.kg^{-1}$ i.p.) was attenuated ($F=29.63$, $dfn=4$, $dfd=35$ $P<0.05$) by oxycodone pretreatment. **E** shows the depression of respiration induced by a challenge dose of oxycodone (3 $mg.kg^{-1}$i.p.) in mice that had received prolonged medium oxycodone treatment. In **F** the area under the curve (AUC) for the percentage change in minute volume in **E** has been determined for each individual animal before the mean AUC has been calculated and compared to that observed in animals that had not received prolonged oxycodone treatment. The depression of respiration induced by the challenge dose of oxycodone (3 $mg.kg^{-1}$i.p.) was attenuated ($F=36.99$, $dfn=2$, $dfd=21$ $P>0.05$ by oxycodone pretreatment. Data were analysed using a One-way ANOVA with Bonferroni's comparison. *p<0.05, ns non significant; N=7 for all groups. In all graphs data are expressed as mean ± SEM.

Figure 4. Reversal by ethanol, pregabalin and calphostin C of tolerance to respiratory depression induced by prolonged treatment with oxycodone. A. In mice that received prolonged treatment with medium dose oxycodone, administration of ethanol (EtOH: 0.3 $g.kg^{-1}$) ($F=21.91$, $dfn=1$, $dfd=24$, $P<0.05$), pregabalin (PG: 20 $mg.kg^{-1}$) ($F=32.58$, $dfn=1$, $dfd=24$

P<0.05) or calphostin C (CC: 45 µg.kg^{-1}) (F=15.99, dfn=1, dfd=24 P<0.05) with the acute morphine challenge resulted in greater depression of respiration than in mice challenged with morphine alone. Data are expressed as mean ± SEM and were compared using a two-way ANOVA with Bonferroni's comparison. * indicates p<0.05 compared to medium oxycodone treatment plus saline/morphine challenge. N=7 for all groups. **B.** Effect of ethanol (0.3 g.kg^{-1}) (F=46.99, dfn=1, dfd=24 P<0.05), pregabalin (20 mg.kg^{-1}) (F=0.99, dfn=1, dfd=24 p>0.05) or calphostin C (45 µg.kg^{-1}) (F=46.94, dfn=1, dfd=24 P<0.05) in mice that had undergone the high dose oxycodone treatment. In mice receiving both ethanol and calphostin C or ethanol and pregabalin there was no greater effect than that seen with either drug alone i.e. additivity was not observed. In each graph the area under the curve (AUC) for the percentage change in minute volume has been determined for each individual animal before the mean AUC has been calculated. Data are expressed as mean ± SEM and were compared using a two-way ANOVA with Bonferroni's comparison. Statistical comparisons not indicated by a bar in B are as follows: * p<0.05 and ns p>0.05 compared to saline/morphine challenge; $ p<0.05 compared to the ethanol + morphine challenge group. N=7 for all groups.

Figure 1

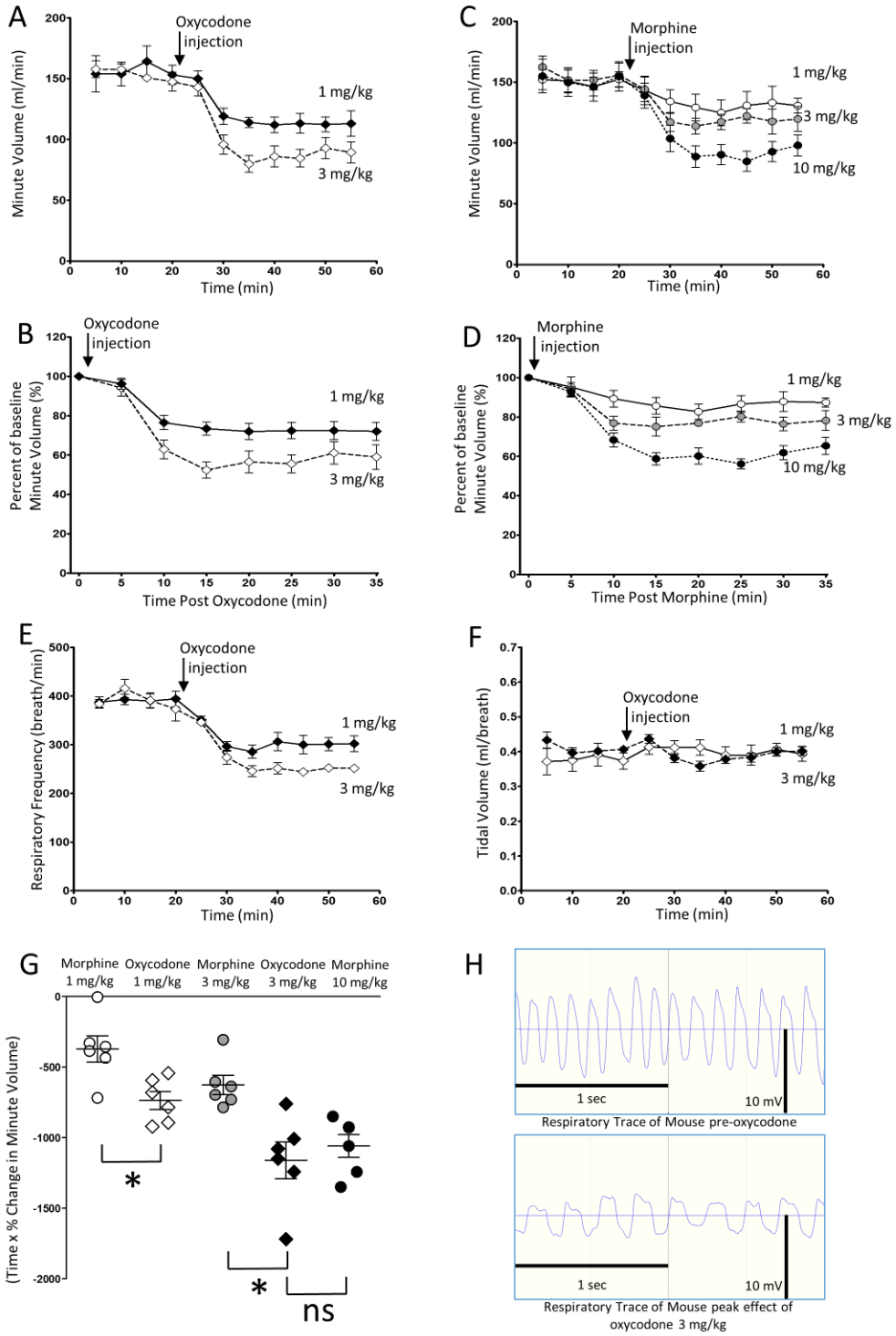

A
Oxycodone injection
1 mg/kg
3 mg/kg
Minute Volume (ml/min)
Time (min)
C
Morphine injection
1 mg/kg
3 mg/kg
10 mg/kg
Minute Volume (ml/min)
Time (min)
B
Oxycodone injection
1 mg/kg
3 mg/kg
Percent of baseline Minute Volume (%)
Time Post Oxycodone (min)
D
Morphine injection
1 mg/kg
3 mg/kg
10 mg/kg
Percent of baseline Minute Volume (%)
Time Post Morphine (min)
E
Oxycodone injection
1 mg/kg
3 mg/kg
Respiratory Frequency (breath/min)
Time (min)
F
Oxycodone injection
1 mg/kg
3 mg/kg
Tidal Volume (ml/breath)
Time (min)
G
Morphine 1 mg/kg
Oxycodone 1 mg/kg
Morphine 3 mg/kg
Oxycodone 3 mg/kg
Morphine 10 mg/kg
AUC (Time x % Change in Minute Volume)
*
*
ns
H
1 sec
10 mV
Respiratory Trace of Mouse pre-oxycodone
1 sec
10 mV
Respiratory Trace of Mouse peak effect of oxycodone 3 mg/kg

Figure 2

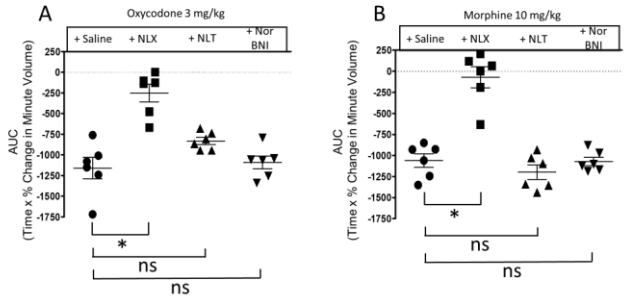

A
Oxycodone 3 mg/kg
+ Saline
+ NLX
+ NLT
+ Nor BNI
AUC (Time x % Change in Minute Volume)
250
0
-250
-500
-750
-1000
-1250
-1500
-1750
*
ns
ns
B
Morphine 10 mg/kg
+ Saline
+ NLX
+ NLT
+ Nor BNI
AUC (Time x % Change in Minute Volume)
250
0
-250
-500
-750
-1000
-1250
-1500
-1750
*
ns
ns

Figure 3

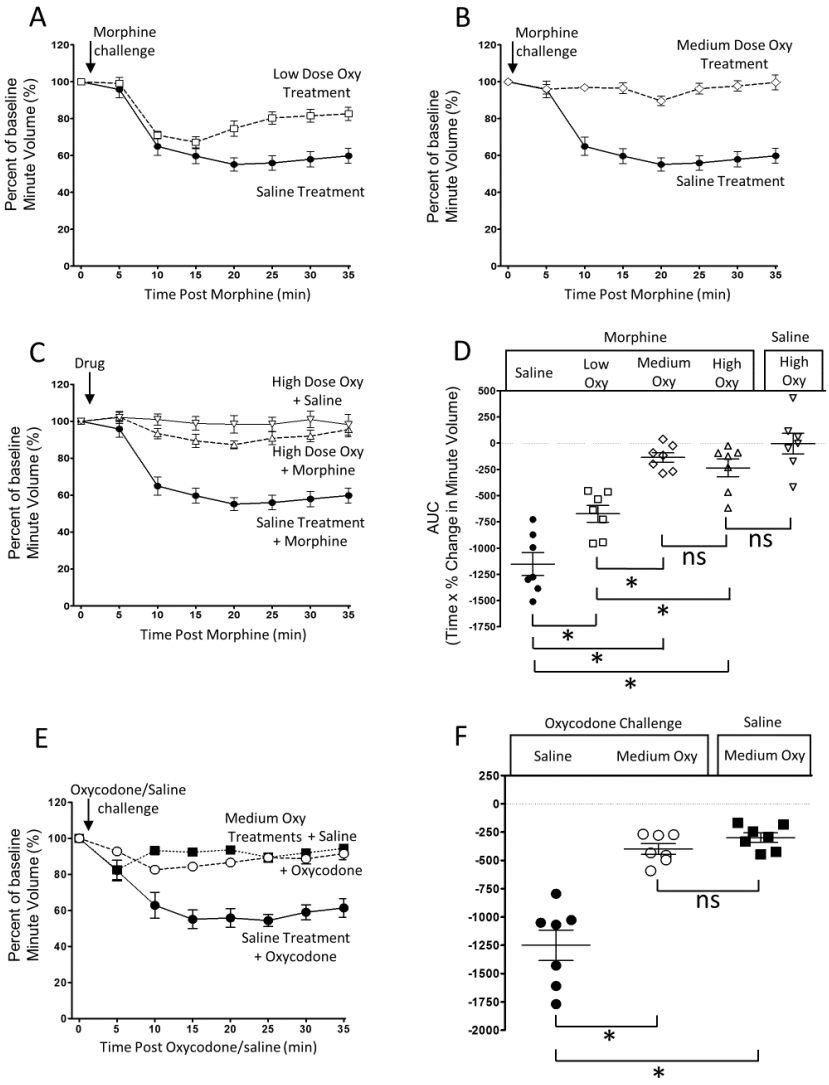
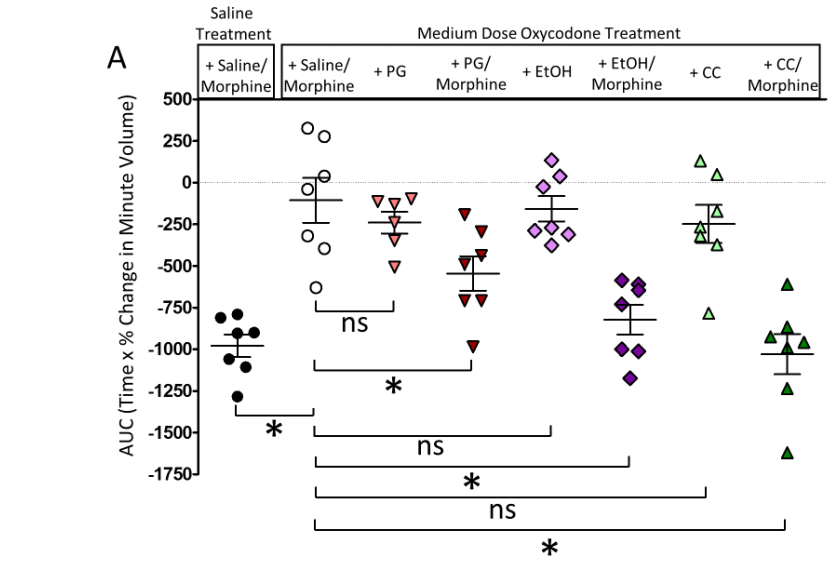
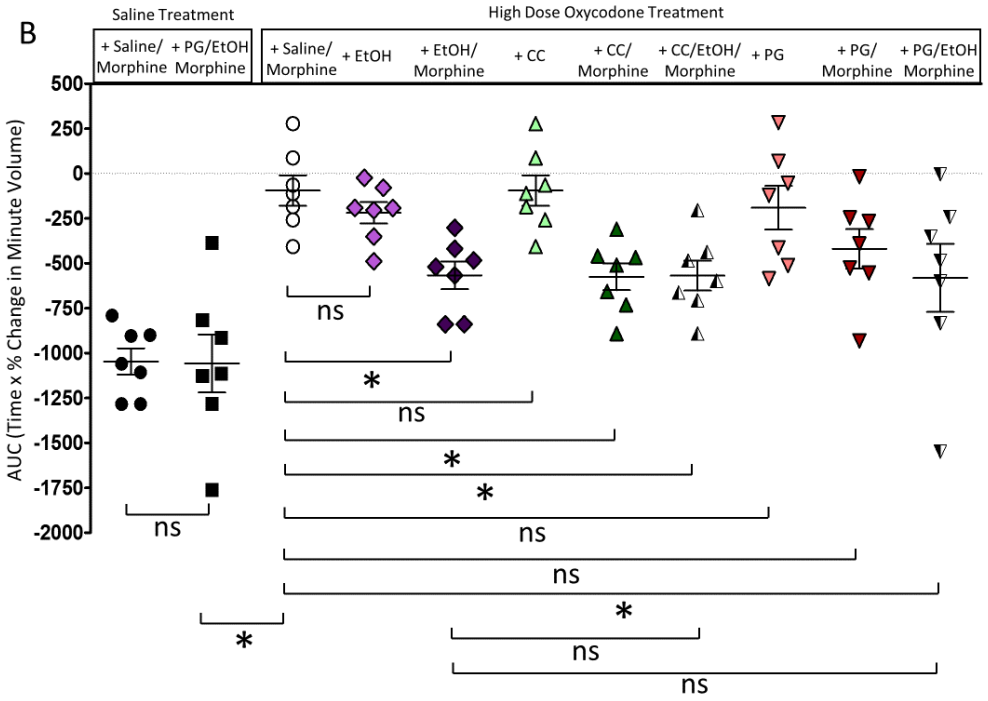

A
Morphine challenge
Low Dose Oxy Treatment
Saline Treatment
Percent of baseline Minute Volume (%)
Time Post Morphine (min)
B
Morphine challenge
Medium Dose Oxy Treatment
Saline Treatment
Percent of baseline Minute Volume (%)
Time Post Morphine (min)
C
Drug
High Dose Oxy + Saline
High Dose Oxy + Morphine
Saline Treatment + Morphine
Percent of baseline Minute Volume (%)
Time Post Morphine (min)
D
Morphine
Saline
Saline
Low Oxy
Medium Oxy
High Oxy
High Oxy
AUC (Time x % Change in Minute Volume)
ns
ns
*
*
*
*
*
E
Oxycodone/Saline challenge
Medium Oxy Treatments + Saline
+ Oxycodone
Saline Treatment + Oxycodone
Percent of baseline Minute Volume (%)
Time Post Oxycodone/saline (min)
F
Oxycodone Challenge
Saline
Medium Oxy
Saline
Medium Oxy
ns
*
*

Figure 4


A
Saline Treatment
Medium Dose Oxycodone Treatment
+ Saline/ Morphine
+ Saline/ Morphine
+ PG
+ PG/ Morphine
+ EtOH
+ EtOH/ Morphine
+ CC
+ CC/ Morphine
AUC (Time x % Change in Minute Volume)
500
250
0
-250
-500
-750
-1000
-1250
-1500
-1750
ns
*
*
ns
*
ns
*


B
Saline Treatment
High Dose Oxycodone Treatment
+ Saline/ Morphine
+ PG/EtOH Morphine
+ Saline/ Morphine
+ EtOH
+ EtOH/ Morphine
+ CC
+ CC/ Morphine
+ CC/EtOH/ Morphine
+ PG
+ PG/ Morphine
+ PG/EtOH Morphine
AUC (Time x % Change in Minute Volume)
500
250
0
-250
-500
-750
-1000
-1250
-1500
-1750
-2000
ns
ns
*
ns
*
*
ns
ns
ns
*
*
ns
ns