A non-rewarding, non-aversive buprenorphine/naltrexone combination attenuates drug-primed reinstatement to cocaine and morphine in rats in a conditioned place preference paradigm

by

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ABSTRACT

Concurrent use of cocaine and heroin is a major public health issue, with no effective relapse prevention treatment currently available. To this purpose, a combination of buprenorphine and naltrexone, a mixed very-low efficacy mu-opioid receptor agonist / kappa-opioid receptor antagonist / NOP receptor agonist, was investigated. The tail withdrawal and the conditioned place preference assays in adult Sprague Dawley rats were used to show that naltrexone dose-dependently blocked the mu-opioid receptor agonism of buprenorphine. Furthermore, in the conditioned place preference assay, a combination of 0.3 mg/kg buprenorphine and 3.0 mg/kg naltrexone was aversive. A combination of 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone was neither rewarding nor aversive, but still possessed mu-opioid receptor antagonist properties. In the conditioned place preference extinction and reinstatement method, a combination of 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone completely blocked drug-primed reinstatement in cocaine-conditioned rats (conditioned with 3 mg/kg cocaine, drug prime was 3 mg/kg cocaine), and attenuated drug-primed reinstatement in morphine-conditioned rats (conditioned with 5 mg/kg morphine, drug prime was 1.25 mg/kg morphine). These data add to the growing evidence that a buprenorphine/naltrexone combination may be protective against relapse in a polydrug abuse situation.

Key words: addiction; buprenorphine; cocaine; morphine; naltrexone; reinstatement

INTRODUCTION

There is currently no medication licensed in Europe or the US for treatment of cocaine dependence, and whilst there are treatments available for opioid dependence, no single treatment is effective for everyone. As many users of crack cocaine are also dependent on heroin, a relapse prevention medication that is effective in the polydrug user population would be a notable step forward.

In this paper, the ability of a combination of buprenorphine and naltrexone to inhibit reinstatement of morphine and cocaine conditioned place preference was investigated. Buprenorphine is a partial agonist at the mu-opioid receptor, an antagonist at the kappa-opioid receptor and a partial agonist at the nociceptin (NOP) receptor (Huang *et al.* 2001). Buprenorphine, like methadone, is widely used as a substitution therapy for treatment of opioid addicts (Maremmani and Gerra 2010), but clinical studies have shown that buprenorphine, but not methadone, is also effective in reducing cocaine use (Kosten, Kleber & Morgan 1989). Naltrexone is an antagonist at both mu- and kappa-opioid receptors (Giordano, Nock & Cicero 1990) and has been shown to reduce both opioid (Comer *et al.* 2006) and cocaine use (Schmitz *et al.* 2001).

Encouraging results have been observed in two clinical trials using a buprenorphine/naltrexone combination therapy (Rothman *et al.* 2000; Gerra, Fantoma & Zaimovic 2006); significant reduction of both heroin and cocaine use was demonstrated. Currently, buprenorphine is licensed as an opioid substitution therapy, but is in itself rewarding via activation of the mu-opioid receptor (Greenwald *et al.* 2007). Combination of buprenorphine with sufficient naltrexone can block buprenorphine's mu-opioid receptor agonism (Dum and Herz 1981; McAleer *et al.* 2003) thus increasing regulatory acceptability, and feasibility of its use in cocaine addicts. Naltrexone is itself licensed as an abstinence-promoter but treatment success is hindered by low compliance. Naltrexone provides no reinforcement or pleasure, but, as a mu-opioid receptor antagonist, is likely to block rewards caused by release of endogenous opioid peptides (Kirchmayer *et al.* 2002;

Mucha, Millan & Herz 1985). Indeed, in laboratory animals, naltrexone alone has been shown to be aversive at high doses (Parker and Rennie 1992, Suzuki *et al.* 1992).

Thus, the combination of buprenorphine and naltrexone may be neither rewarding nor aversive, but still an effective anti-addiction therapy. In addition to effects on mu-opioid receptors, the buprenorphine/naltrexone combination acts as an antagonist at the kappa-opioid receptor. Comorbid affective disorders can emerge on cessation of use of drugs of abuse (Gerra *et al.* 2006), and antagonism of the kappa-opioid receptor appears to counter depression in rodents (Mague *et al.* 2003; Beardsley *et al.* 2005). Further, the kappa-opioid system and its endogenous agonist dynorphin have been shown to mediate many stress responses; in preclinical studies, inhibition or genetic ablation of kappa-opioid receptors has been shown to inhibit stress-induced, but not drug-prime-induced, reinstatement to cocaine-seeking behavior (reviewed by Bruchas, Land & Chavkin 2010).

Another component of the pharmacology of a buprenorphine/naltrexone combination is to act as a partial agonist at the NOP receptor. Selective NOP agonists are neither rewarding nor aversive (Le Pen *et al.* 2002) and, although the mechanism is poorly understood, they have been shown in rodents to oppose the effects of cocaine and morphine (Kotlinska *et al.* 2002; Sakoori and Murphy 2004), inhibiting drug-primed reinstatement of morphine conditioned place preference (CPP) (Shoblock, Wichmann & Maidment 2005). The effect of NOP agonists on reinstatement of cocaine CPP has not been studied to date.

Overall, a mixed very-low efficacy mu-opioid receptor agonist / kappa-opioid receptor antagonist / NOP receptor agonist is a desirable target as a novel anti-addiction therapy (McCann 2008). The reduction in cocaine use observed in the studies carried out by Rothman and Gerra could be via antagonism of mu-opioid receptors attenuating the positive reinforcing effects of cocaine (Bilsky *et al.* 1992), via antagonism of kappa-opioid receptors conferring stress resilience (Redila and Chavkin 2008), or via more generalized anti-addictive effects of agonism of the NOP receptor

(Shoblock *et al.* 2005; Kuzmin *et al.* 2007). Alternatively, it could simply be a consequence of reduction in heroin use (these two drugs of abuse are often used by addicts to 'complement' each other). Therefore, the effect of buprenorphine/naltrexone on the effects of cocaine in a controlled experiment has been assessed here. The aims of this study were to determine the ratio of buprenorphine/naltrexone that is neither rewarding nor aversive, and then to evaluate how this drug combination can inhibit drug-primed reinstatement of both morphine- and cocaine-induced conditioned place preference.

MATERIALS AND METHODS

Subjects

All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and the University of Bath's ethical review documents. Male Sprague Dawley rats (Charles River, UK) were used; 260-420g (7-11 weeks old) for tail withdrawal and rat vas deferens experiments, 250-320g (7-9 weeks old) for conditioned place preference experiments. All rats were housed four per cage with *ad libitum* access to food and water and maintained on a 12:12 h light-dark cycle (lights on 07:00, lights off 19:00).

Tail withdrawal assay

A water bath (Grant Instruments, UK) was maintained at 52ºC. The rats were held firmly in a vertical position, and lowered until the distal third of the tail was in the water. The time taken for the rat to withdraw the tail was recorded. A 20 second cut-off was imposed to avoid tissue damage. All rats were opioid naïve, and were not reused.

Measurement of receptor affinity – rat vas deferens

Rats were killed using CO_2 and vasa deferentia were excised and suspended in a siliconized tissue bath (3 ml volume) under 0.5 g tension in Krebs-bicarbonate solution (composition (mM): NaCl 118, KCl 4.74, $CaCl_2$ 2.50, KH_2PO_4 1.19, $MgSO_4$ 1.20, $NaHCO_3$ 25, glucose 11, bubbled with 95% O_2 / 5% CO_2, maintained at 37ºC). Nerve-evoked muscle contractions were induced with single square pulses (0.1 ms duration, 0.1 Hz, supramaximal voltage) and measured isometrically with 'LabChart' software (AD Instruments). Cumulative concentration response curves to the selective mu-opioid receptor agonist, [D-Ala^2,NMe-Phe^4,Gly-ol^5]-enkephalin (DAMGO; concentration increased at 5-minute intervals) were constructed in the absence then presence of buprenorphine or naltrexone.

In individual tissues, EC_{50} values for DAMGO in the absence and presence of buprenorphine or naltrexone were derived by fitting data to a non-linear regression curve-fit (GraphPad Prism) using the equation:

Effect = baseline + (Emax-baseline)/(1+10^((LogEC50 - X)*HillSlope))

where X is the agonist concentration.

For naltrexone, a Schild plot was constructed, deriving a pA_2 value (equivalent to the log K_B value). As buprenorphine has been shown to be pseudo-irreversible, K_B values were obtained using a single concentration of buprenorphine (1 nM) and the Schild equation.

Calculations of relative receptor occupancy were derived using the following equation:

% occupancy of drug A = $100 * ([A]/K_A) / (([A]/K_A) + ([B]/K_B) + 1)$

Blood and brain tissue concentrations

Plasma sample preparation - Blood samples (30-minute post-injection) were centrifuged (3300 rpm, 10 minutes), and plasma recovered. Plasma samples were basified using ammonium hydroxide solution (pH 10) and loaded onto a solid phase extraction (SPE) cartridge (Waters Oasis HLB) previously conditioned with 1 ml methanol and 1 ml water. The cartridge was washed with 1 ml 2% methanol in ammonium hydroxide solution (pH 10), rinsed with water, then the analyte was eluted with 440 µl 60% methanol containing 2% acetic acid. The sample was filtered (nylon 0.45 µm syringe filter) before injection into the LC-MS. Samples were injected undiluted to analyse buprenorphine, then diluted 1:1 with water to analyse naltrexone.

Brain tissue sample preparation - The brain tissue (30-minute post-injection) was prepared using the same SPE process as the plasma samples. Prior to the SPE process water was added (1.8 ml/gram of brain tissue) to facilitate homogenisation (Tissue Master 240, OMNI International, US), the sample was centrifuged, and the supernatant fluid was collected.

Buprenorphine and norbuprenorphine LC-MS method - Separation was performed using a GeminiNX column (3µm C18 110A 50 x 2mm) from Phenomenex, maintained at 25°C, on a Shimadzu LC-2010AHT HPLC. The mobile phase was 18:82 acetonitrile: 0.1% acetic acid at a flow rate of 0.2 ml/min. 30µl of sample was injected.

The retention times for buprenorphine and norbuprenorphine were 9 and 3 minutes; masses per charge were 468 and 414. Standards were prepared in SPE eluent. Limit of quantitation was 0.18 ng/ml for buprenorphine and 0.8 ng/ml for norbuprenorphine.

Naltrexone and 6ß-naltrexol LC-MS method - The method for analysis of naltrexone and 6ß-naltrexol was adapted from Valiveti (2004). Separation was performed using a Symmetry column (5µm C18 110A 150 x 2.1 mm) from Waters, maintained at 23°C, on a Shimadzu LC-2010AHT HPLC. The mobile phase was 12:88 acetonitrile: 0.1 % ammonium acetate at a flow rate of 0.25 ml/min. 30µl of sample was injected.

The retention times for naltrexone and 6ß-naltrexol were 4 and 3 minutes; masses per charge were 342 and 344. Standards were prepared in SPE eluent. Limit of quantitation was 3.8 ng/ml for naltrexone and 0.28 ng/ml for 6ß-naltrexol.

Conditioned place preference apparatus

CPP boxes (Tracksys, UK) were three-chambered shuttle boxes comprising a small central compartment (10 x 10 cm) where rats were placed at the start of a test session, and two larger compartments (40 x 40 cm), one with horizontal black and white stripes, and one with vertical black and white stripes. Floors were made of stainless steel sheeting with punched-out shapes (circles, 12 mm hole, and squares, 10 mm hole) resulting in distinct textures (Novametals, UK). Removable partitions allowed the boxes to be used either to restrict the rats to a particular compartment for conditioning, or to allow the rats to be ‘free-to-explore’ during a test session. Experiments were performed between 8 am and 5 pm under dim white light (light intensity approx.

15 lux). During all test sessions, the time each rat spent in each compartment was recorded using EthoVision XT (Tracksys, UK) tracking software.

Rats were assigned to treatment groups randomly, and the pairing was counterbalanced (i.e., within each cohort, equal numbers of rats were always drug-paired to each compartment type). Groups were organised such that mean baseline % preferences were close to zero.

CPP. Data throughout are presented after multiplying by a correction factor. The correction factor was calculated by dividing duration of test (seconds) by total time spent (seconds) in the two large compartments. This factor is used to proportionally divide the time spent in the neutral central compartment between the two conditioning compartments.

% preference was calculated using the corrected data such that if a rat spent equal time in the drug-paired compartment and the saline-paired compartment, the preference score would be zero %. If a rat spent no time in the saline-paired compartment, the preference score would be 100 %.

Individual rats whose baseline preference was >16.7 or <-16.7 were excluded.

Drugs and chemicals

Naltrexone hydrochloride dihydrate was from Fluka (UK). Saline (sodium chloride 0.9%) was from Dechra (UK). Buprenorphine hydrochloride was prepared in-house. Cocaine hydrochloride and morphine sulphate were from MacFarlan Smith (UK). All in vivo injections were intraperitoneal (1 ml/kg).

DAMGO was from Bachem (UK). Sigmacote®, buffer components and mobile phase components were from Sigma (UK).

PROCEDURES

Using naltrexone to block the rewarding effects of buprenorphine

Two separate assays, tail withdrawal and CPP, were used to establish the dose of naltrexone required to block the mu-opioid receptor agonism of buprenorphine.

Tail withdrawal assay. Five baseline measurements were taken, one immediately after another, for each rat. 0.3 mg/kg buprenorphine was then administered in combination with either 0, 0.3 or 1.0 mg/kg naltrexone (n = 5, 4, 7). Following injection of the drug, measurements were taken once every 7.5 minutes, up to 60 minutes. Data collected at the 60 minute time-point was used in subsequent analyses. Baseline tail-withdrawal time was taken as the mean of the last 2 baseline measurements. For each rat, analgesia was quantified as tail-withdrawal time post-drug treatment minus baseline measurement. Data were analysed using one-way ANOVA with the Bonferroni post-test.

CPP assay. Rats were conditioned with buprenorphine (0.3 mg/kg) administered in combination with either 0, 0.3, 1 or 3 mg/kg naltrexone (n = 16, 8, 8, 16). On day 1, rats had a 15 minute exploratory session; on day 4, they had a 15 minute baseline preference test. A % preference was obtained. On days 5-8 and 11-14, the rats received drug or saline on alternate days, thus, each rat had 4 drug injections and 4 saline injections. Injections were administered at least 24 hours apart to ensure that the effects of buprenorphine had dissipated before subsequent saline injections. Following injections, the rats were immediately confined to a compartment for 40 minutes. On day 15, % preference was obtained exactly as for baseline preference; i.e., the preference for each drug treatment was measured by recording the time spent in the drug-paired chamber in a free-to-explore test lasting 15 minutes. To assess conditioning, a 1-tailed Wilcoxon matched pairs signed-rank test was used (each group's % preference after drug treatment compared to its baseline).

Measuring mu-opioid receptor antagonism of a buprenorphine/naltrexone combination

Five baseline measurements were taken. Latency to withdrawal was measured following administration of 10 mg/kg morphine only (n = 4), and when both 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone (n = 5) or 1.0 mg/kg naltrexone alone (n = 7) were administered 30 minutes

prior to the morphine. Following injection of the morphine, measurements were taken once every 5 minutes, up to 30 minutes. Data collected at the 30 minute time point was used in subsequent analyses.

Effects of buprenorphine/naltrexone on reinstatement of cocaine and morphine conditioned place preference

To test the ability of a buprenorphine/naltrexone combination to block drug-primed reinstatement, a conditioned place preference extinction and reinstatement method was established (Figure 1).

Rats had one 15 minute exploratory session and one 15 minute baseline preference test. Animals were conditioned using either 3 mg/kg cocaine or 5 mg/kg morphine, receiving drug and saline on the same day (at least 4 hours apart) for 3 consecutive days. Immediately after injection, rats were confined to a particular compartment (drug-paired or saline-paired) in the CPP box (for 20 minutes or 40 minutes, cocaine and morphine respectively). Following conditioning, % preference was obtained exactly as for baseline preference. Individual rats which showed less than a 30-second increase for drug-paired side over their baseline preference during the post-conditioning test were excluded from extinction and reinstatement. To assess conditioning *before* exclusions, a 1-tailed Wilcoxon matched pairs signed-rank test was used (each group's % preference after drug treatment compared to its baseline).

Four cohorts of rats were used: cocaine-conditioned control (n = 20), morphine-conditioned control (n = 24), cocaine-conditioned buprenorphine/naltrexone treatment (n = 16), and morphine-conditioned buprenorphine/naltrexone treatment (n = 16).

For all cocaine-conditioned rats, extinction was achieved by reconditioning. This involved injection of saline followed by confinement to a compartment for 20 minutes, twice a day for 4 days (4 hours apart). The rats were alternated daily as to whether they were placed in the previously drug-

paired compartment or the previously saline-paired compartment morning or afternoon. Extinction was confirmed by a 15 minute free-to-explore test (day 15, see Figure 1).

For the morphine-conditioned rats, two styles of extinction were used: a reconditioning style of extinction (saline injection with 15 minutes confinement in each compartment, as above), and a retesting style of extinction (daily retesting, 15 minutes per test). Of the animals subsequently tested for reinstatement, 5 rats in the control group underwent extinction training using the reconditioning style, and 9 underwent extinction using the retesting style. All rats in the buprenorphine/naltrexone treatment group underwent extinction training using the reconditioning style.

Extinction training was deemed complete if group mean preference was <5%. For the retesting style of extinction, this was taken from the average of 2 consecutive days. Following extinction, rats were administered a priming dose of 3 mg/kg cocaine or 1.25 mg/kg morphine. The control groups received a saline injection 10 minutes prior to drug priming and the treatment groups received a buprenorphine/naltrexone injection (0.3 and 1 mg/kg respectively) 10 minutes prior to drug priming. Following administration of the priming dose, rats were placed immediately into the CPP boxes and were free-to-explore during a 30-minute test.

Conditioning and reinstatement were assessed using the Friedman test followed by Dunn's multiple comparison test (each group's % preference compared to its baseline). A Mann-Whitney U test was used to compare the % preference during the reinstatement test for rats that had undergone a retesting style of extinction and for rats that had undergone a reconditioning style of extinction.

It was observed that in the control groups, conditioned place preference behavior emerged at 13-15 minutes of the reinstatement test period in cocaine-conditioned rats; however, in the morphine-conditioned rats, drug-seeking was evident from the start of the reinstatement test period and then

diminished somewhat after 15 minutes (data not shown). The data from 0-30 minutes of the reinstatement test were used for the cocaine-conditioned rats, whereas, the data from 0-15 minutes of the test were used for the morphine-conditioned rats. It is interesting that these findings are in contrast to Mueller and Stewart (2000) and Mueller, Perdikaris & Stewart *(*2002) who observed gradual emergence of drug-seeking over the course of the reinstatement test in both cocaine- and morphine-conditioned rats.

RESULTS

Using naltrexone to block the rewarding effects of buprenorphine

Tail withdrawal assay. Tail withdrawal is a commonly used assay of analgesia, and was used here as an indirect measure of mu-opioid receptor agonism. A dose of 0.3 mg/kg buprenorphine was selected because previous studies in rats have indicated that this dose is rewarding in the CPP assay (Suzuki *et al.* 1992; Rowlett, Gibson & Bardo 1994; Tzschentke 2004), and can elicit measureable mu opioid receptor-mediated analgesia (Lutfy *et al.* 2003). Figure 2 (panel A) shows that 0.3 mg/kg buprenorphine elicited marked analgesia in this experimental set-up. This can be seen by the increase in time taken to withdraw the tail compared to baseline. A clear dose-dependent effect of naltrexone countering buprenorphine-induced analgesia was observed.

CPP assay of rewarding effects of buprenorphine*.* Group mean baseline preferences were 0 ± 2, 2 ± 5, 2 ± 2 and 0 ± 2 % (mean ± SEM). One rat in the 0.3 mg/kg buprenorphine group and one rat in the 0.3 mg/kg buprenorphine and 0.3 mg/kg naltrexone group were excluded for having a preference at baseline.

0.3 mg/kg buprenorphine elicited drug-seeking behavior in this set-up (Figure 2, panel B). This can be seen as a significant increase in time spent in the drug-paired compartment compared to baseline. As observed in the tail withdrawal assay, a clear dose-dependent effect of naltrexone was observed; naltrexone countered the rewarding effects of buprenorphine to the extent that co-administration of 3.0 mg/kg naltrexone actually elicited aversion (rats spent significantly more time in the saline–paired compartment compared to baseline test).

There was good agreement between the results of the tail withdrawal and the CPP assays; taken together, it was clear that, in these rats, 1.0 mg/kg naltrexone blocked the mu-opioid receptor agonism of 0.3 mg/kg buprenorphine. This finding fits with earlier data (Walker *et al.* 1994) which showed that 1.0 mg/kg naltrexone blocked the subjective effects of 0.3 mg/kg buprenorphine in a discrimination assay in rats. A combination of 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone

was therefore selected for use in subsequent behavioral experiments.

Measuring mu-opioid receptor antagonism of a buprenorphine/naltrexone combination

After establishing that 1.0 mg/kg naltrexone was sufficient to block the mu-opioid receptor agonism of 0.3 mg/kg buprenorphine, we tested whether this combination would show mu-opioid receptor antagonism. 10 mg/kg morphine elicited a measurable analgesia (Figure 3).

Clear mu-opioid receptor antagonism was observed following administration of buprenorphine/naltrexone (morphine-induced analgesia decreased from 16 ± 0 to 3 ± 1 seconds, mean ± SEM). Administration of naltrexone alone blocked morphine-induced analgesia to a greater extent than did the combination.

CPP extinction and reinstatement model

The CPP extinction and reinstatement method is frequently used to study the effects of potential relapse prevention treatments (reviewed by Aguilar, Rodriguez-Arias & Minarro 2009). This method was therefore used to observe drug-primed reinstatement in cocaine- and morphine-conditioned rats, and the effects thereon of pre-treatment with a buprenorphine/naltrexone combination. Table 1 shows the number of rats used in the experiments, the number of rats excluded, and the time taken to reach extinction. Conditioning was statistically significant in each cohort before individual rats were excluded for insufficient conditioning.

Control groups. Figure 4 shows the data for the control groups (panels A and B). The % preference post-conditioning and following a drug prime was significantly different from baseline for both drugs. In other words, reinstatement of drug-seeking was successfully attained in both control groups. As there was no significant difference in % preference during the reinstatement test between the morphine-conditioned rats, which underwent the two styles of extinction training

used (reconditioning 11 ± 7 % n = 5, retesting 19 ± 9 % n = 9, mean ± SEM), pooled data are shown.

Buprenorphine/naltrexone treatment groups. Figure 4 shows the data for the buprenorphine /naltrexone treated groups (panels C and D); the effect of the combination on drug-primed reinstatement in cocaine- and morphine-conditioned rats is clear. Preference during reinstatement test was not significantly different from baseline for either drug. In the cocaine-conditioned rats, the buprenorphine/naltrexone treatment completely blocked reinstatement (preference score of -9 ± 5 % mean ± SEM compared to 11 ± 5 in the control group). In the morphine-conditioned rats, the buprenorphine/naltrexone treatment attenuated the preference observed following administration of a drug prime (preference score of 6 ± 10 %, mean ± SEM compared to 16 ± 6 in the control group).

Receptor occupancy of buprenorphine and naltrexone

Having demonstrated that a combination of 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone is neither rewarding nor aversive, and is capable of blocking drug-primed reinstatement to cocaine- and morphine-seeking, we next estimated the relative receptor occupancies of both drugs.

Receptor affinity values were determined in isolated tissue (rat vas deferens) and $\log K_B$ values were -9.38±0.12 for buprenorphine (ie. K_B = 0.41 nM) and -8.90±0.12 for naltrexone (ie. K_B = 1.26 nM) (Figure 5). Plasma and brain concentrations were measured 30 minutes after administration of buprenorphine (0.3 mg/kg) and naltrexone (1.0 mg/kg) (Table 2). From these data, receptor occupancy levels of each could be estimated.

These data suggest that >95% of all available mu-opioid receptors are occupied either by buprenorphine or naltrexone when administered at these doses, which, as Figure 3 shows, is sufficient to block morphine-induced analgesia. Furthermore, when buprenorphine (0.3 mg/kg) is co-applied with naltrexone (1.0 mg/kg), buprenorphine only occupies ~40% of available receptors,

and at this receptor occupancy level there is not sufficient mu-opioid receptor activation to produce either analgesic or rewarding effects (see Figure 2). In addition, similar experiments (data not shown) were performed to determine the affinity of buprenorphine at kappa-opioid receptors. The K_B value of buprenorphine at kappa-opioid receptors (0.6 nM) means that at the observed brain concentrations, >95% of all available kappa-opioid receptors would be occupied by buprenorphine or naltrexone.

DISCUSSION

Data from both the tail withdrawal and the conditioned place preference assays showed that 0.3 mg/kg buprenorphine alone displayed marked mu-opioid receptor agonism and that naltrexone dose-dependently blocked the mu-opioid receptor agonism in both assays. A critical feature for a potential anti-addiction therapy is that the therapy itself is neither rewarding (and therefore would itself have an abuse liability) nor aversive (and could reduce compliance or effectiveness (Greenwald *et al.* 2007; Manlandro 2007; Nutt 2010)). In our study, although 0.3 mg/kg buprenorphine alone was rewarding, as seen by conditioned place preference, when buprenorphine was administered in combination with 1.0 mg/kg naltrexone the combination was neither rewarding nor aversive. If a higher dose of naltrexone (3.0 mg/kg) was used, the combination became aversive. Previous studies have shown that mu-opioid receptor antagonism can induce conditioned place aversion, an effect attributed to blockade of endogenous opioid activity (Mucha *et al.* 1985).

We generated estimates of relative receptor occupancies following buprenorphine/naltrexone combination (0.3 / 1 mg/kg). >95% of available mu-opioid receptors were estimated to be occupied, 40% of these by buprenorphine. One caveat is that we could only measure absolute brain levels rather than free concentrations of each drug. A high proportion of the compounds may be in the lipid compartment and unavailable for receptor binding (buprenorphine being particularly lipophilic). In vivo autoradiography studies have found in vivo K_D values for buprenorphine and naltrexone at mu-opioid receptors to be approximately 23 and 30 μg/kg respectively (Richards and Sadée 1985; Höllt *et al.* 1975). Although brain and plasma concentrations were not measured in those studies, if we extrapolate from our empirical brain concentrations, this suggests brain concentrations for both drugs in those studies would be approximately 10x higher than in vitro K_B values. Hence, approximately 10% of both buprenorphine and naltrexone in the brain would be 'free'. If this were the case, this would have a negligible impact on both relative and total receptor occupancy by buprenorphine and naltrexone at the doses used in this study, based on the fact that

the doses used here resulted in brain levels of both drugs far in excess (>100-fold higher) of their K_B values.

The clinical trials carried out by Rothman *et al.* (2000) and Gerra *et al.* (2006) administered naltrexone orally (50mg daily) and buprenorphine sublingually (4mg daily). Although plasma drug concentrations were not performed in those studies, they would likely have been of the order of 4nM buprenorphine and 60nM naltrexone (peak levels) declining to 0.4nM buprenorphine and 3nM naltrexone (at 24 hours) (Everhart *et al.* 1997; Verebey *et al.* 1976), compared to 56nM buprenorphine and 285nM naltrexone in this study. While there are limits to the extent to which our studies in rat can be compared to clinical studies, our data suggest two things for future clinical studies. Firstly, that the ideal buprenorphine:naltrexone plasma concentration ratio is around 1:5, theoretically meaning that relatively higher buprenorphine doses would be more clinically effective. Secondly, higher doses of *both* buprenorphine and naltrexone than those used by Rothman *et al.* (2000) and Gerra *et al.* (2006) may be even more effective clinically, as the combination would result in greater mu and kappa-opioid receptor occupancies.

The ratio of naltrexone to buprenorphine dose in the current study (3:1) is very different to that used in a recent study examining cocaine self-administration in rats (Wee *et al.* 2012) where 0.3 mg/kg naltrexone and 3 mg/kg buprenorphine was predominantly used (1:10). Although different routes of administration were used (subcutaneous in Wee *et al.* 2012; intraperitoneal in the current study), our data would suggest that if more buprenorphine than naltrexone is administered, there would still be a significant mu-opioid receptor agonist response. Indeed, Wee *et al.* (2012) showed that although 0.3 mg/kg naltrexone reduced somatic withdrawal signs and analgesia following 3 mg/kg buprenorphine, there were still significant signs of residual mu-opioid receptor activation. Our study shows that when plasma and brain levels of buprenorphine are around 4-5-fold less than that of naltrexone there is no measurable mu-opioid receptor agonist response.

The reduction in morphine-induced analgesia in the tail withdrawal test following administration of

the combination indicates that the combination has significant mu-opioid receptor antagonist properties. Indeed, when buprenorphine and naltrexone were administered in combination, >95% of available mu-opioid receptors were estimated to be occupied. However, the combination did not counter morphine to the same extent as naltrexone alone. This may be because of very low levels of mu-opioid receptor activation by the buprenorphine/naltrexone combination which, although below the level of functional effect, may be added to with a morphine challenge.

Data generated using the CPP extinction and reinstatement method showed clearly that buprenorphine/naltrexone reduced reinstatement to drug-seeking following a drug prime, in both cocaine- and morphine-conditioned rats. Unexpectedly, the effect was more dramatic in the cocaine-conditioned rats. Whilst there is preclinical data showing that buprenorphine (Kosten, Marby & Nestler 1991; Suzuki *et al.* 1992; Comer *et al.* 1993) and naltrexone (Bilsky *et al.* 1992; Suzuki *et al.* 1992) can reduce the ability to acquire a conditioned place preference to cocaine, the research here goes further. Firstly, the ability of buprenorphine and naltrexone to block reinstatement to cocaine-seeking following extinction was demonstrated, and secondly, the two compounds were administered as a combination that by itself induces neither conditioned place preference nor aversion. Importantly, the observed reduction in cocaine-seeking implies that the decrease in cocaine use reported in clinical trials (Rothman *et al.* 2000; Gerra *et al.* 2006) was probably not a simple consequence of reduced heroin use.

As well as blocking mu-opioid receptors, the combination of buprenorphine and naltrexone used here also acts as a functional kappa-opioid receptor antagonist (>95% estimated occupancy of available kappa-opioid receptors). However, kappa-opioid receptor antagonism is not thought to be effective in blocking drug-primed reinstatement to cocaine (Beardsley *et al.* 2005), only in blocking that which is stress-induced (Beardsley *et al.* 2005; Carey *et al.* 2007; Land *et al.* 2009). Therefore, it may be postulated that the successful blocking of reinstatement to cocaine-seeking observed here was due, at least in part, to some ‘non-kappa’ effects, such as mu-opioid receptor antagonism. Certainly, the opioid system is heavily involved in the hedonic response and the

subsequent reinforcement process of all drugs of abuse, including cocaine (Kalivas and Tang, 2005; Soderman & Unterwald 2008; Le Merrer *et al.* 2009). Whilst NOP activation in mice has been shown to counteract cocaine reward (Bebawy *et al.* 2010) and morphine reward and antinociception (Lutfy *et al.* 2003; Marquez *et al.* 2008; Rutten *et al.* 2011), it is not yet known what dose of buprenorphine would be required for NOP agonism to become relevant to drug-seeking behaviour in rats.

In summary, it has been shown that a combination of 1.0 mg/kg naltrexone and 0.3 mg/kg buprenorphine, administered i.p. in Sprague Dawley rats, is non-rewarding and non-aversive, but results in high occupancy levels of both mu- and kappa-opioid receptors, and so acts as a functional mu/kappa-opioid receptor antagonist. It was then demonstrated that 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone blocked drug-primed reinstatement to cocaine-seeking, and attenuated drug-primed morphine-seeking. These data add to the growing evidence that a buprenorphine/naltrexone combination may be effective in a polydrug abuse situation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS' CONTRIBUTION:

The study was conceived by SMH. CPB was involved in all experimental design. SFC and AT were responsible for the behavioural experiments, and the tissue concentration measurements. IER was responsible for the receptor affinity experiments. CPB and SFC drafted the manuscript. All authors provided critical revision of the manuscript for important intellectual content.

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FIGURE LEGENDS

Figure 1 Schematic of the time course of the conditioned place preference extinction and reinstatement method. Filled-in circles represent cocaine or morphine injections and empty circles represent saline injections (it can be seen that during conditioning the order of injections alternated daily). This schematic shows the reconditioning style of extinction, where each rat received 2 saline injections per day. The arrow indicates that buprenorphine/naltrexone treatment (or saline, in the control groups) was administered 10 minutes prior to the priming dose.

Figure 2 The tail withdrawal assay and the CPP assay show how naltrexone blocks the mu-opioid receptor agonism of buprenorphine in a dose-dependent fashion. **A** Increase from baseline (seconds) in tail withdrawal assay at 60 minute time point (n = 5, 4, 7), mean + SEM. Baseline values were in the range 4 - 6 seconds; * indicates that first and third columns are significantly different from one another $p<0.05$. **B** % preference in CPP assay (n = 15, 7, 8, 16), mean + SEM; * indicates significantly different from baseline $p<0.05$. From left to right: 0.3 mg/kg buprenorphine, 0.3 mg/kg buprenorphine and 0.3 mg/kg naltrexone, 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone, and (conditioned place preference only) 0.3 mg/kg buprenorphine and 3.0 mg/kg naltrexone.

Figure 3 The tail withdrawal assay shows antagonism of morphine by naltrexone, with and without buprenorphine. Data shows measurements taken 30 minutes after administration of 10 mg/kg morphine, and 60 minutes after administration of buprenorphine and/or naltrexone. From left to right, rats received 0 mg/kg buprenorphine (n = 5), 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone (n = 4), and 1.0 mg/kg naltrexone (n = 7). Baseline values were in the range 4 - 5 seconds. Mean + SEM; * indicates significantly different ($p<0.05$) vs. morphine alone group; ** indicates significantly different ($p<0.05$) vs. morphine alone group and vs. 0.3 mg/kg buprenorphine and 1.0 mg/kg naltrexone group.

Table 1 Number of rats used in the experiments, and excluded at each stage, and time taken to reach extinction.

Figure 4 The CPP assay shows the effect of buprenorphine/naltrexone treatment on reinstatement to drug-seeking. % preference of rats (left to right in each panel: baseline, post-conditioning, post-extinction and drug-primed reinstatement). Drug prime was 3 mg/kg cocaine or 1.25 mg/kg morphine. **A** cocaine-conditioned control group (n = 12); **B** morphine-conditioned control group (n = 14). **C** cocaine-conditioned buprenorphine/naltrexone treated group (n = 9); **D** morphine-conditioned buprenorphine/naltrexone treated group (n = 8). Mean + SEM; * indicates significantly different from baseline $p<0.05$. Arrows indicate that buprenorphine/naltrexone treatment (0.3 mg/kg/1.0 mg/kg) was administered 10 minutes prior to the priming dose.

Figure 5 Inhibition of electrically-evoked twitch in rat vas deferens by DAMGO. Effects of DAMGO are inhibited by buprenorphine (bup), **A** data from a single tissue; **B** pooled data from 4 tissues. Effects of DAMGO are inhibited by naltrexone (naltrex), **C** data from a single tissue; **D** pooled data from 5 tissues; **E** Schild plot from data shown in D. Solid line: line of best-fit when slope constrained to 1, dashed line: 95% confidence limits.

Table 2. Plasma and brain concentrations of buprenorphine and naltrexone and estimated receptor occupancies. Buprenorphine and naltrexone were administered simultaneously intraperitoneally. Plasma and brain samples were taken 30 minutes later and buprenorphine and naltrexone levels measured. The primary metabolites of buprenorphine and naltrexone (norbuprenorphine and 6β-naltrexol) were also assayed but were below the limit of quantitation in plasma and brain samples. Using the plasma and brain concentrations, and empirically-determined K_B values of both buprenorphine and naltrexone, receptor occupancy levels were determined. All data shows as mean ± S.E.M.

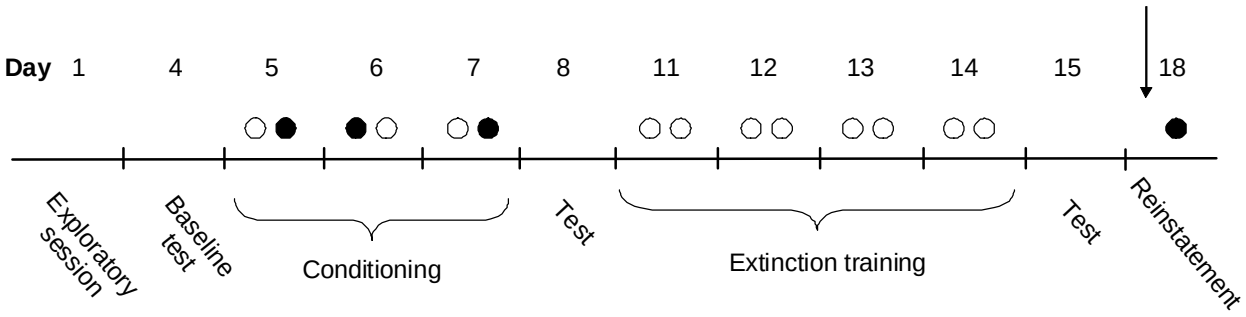
Day 1
4
5
6
7
8
11
12
13
14
15
18
Exploratory session
Baseline test
Conditioning
Test
Extinction training
Test
Reinstatement

FIGURE 1

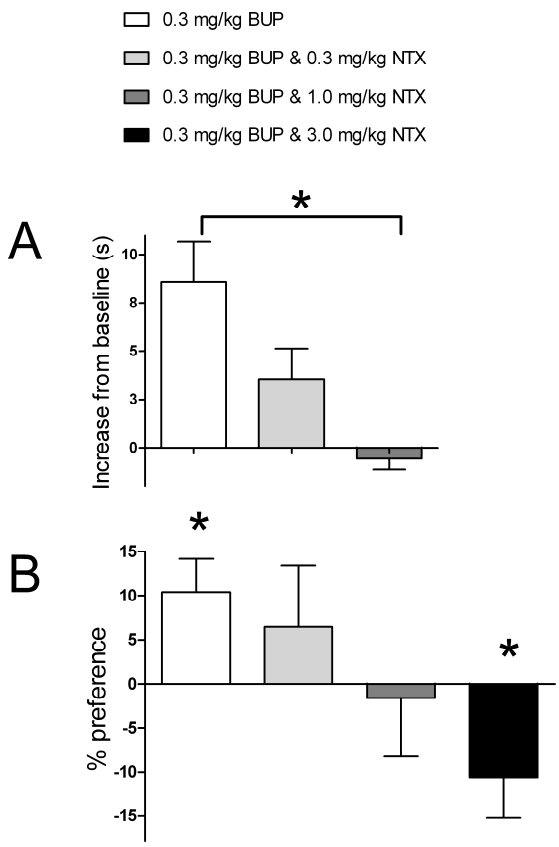
0.3 mg/kg BUP
0.3 mg/kg BUP & 0.3 mg/kg NTX
0.3 mg/kg BUP & 1.0 mg/kg NTX
0.3 mg/kg BUP & 3.0 mg/kg NTX
A
*
Increase from baseline (s)
10
8
5
3
0
B
*
*
% preference
15
10
5
0
-5
-10
-15

FIGURE 2

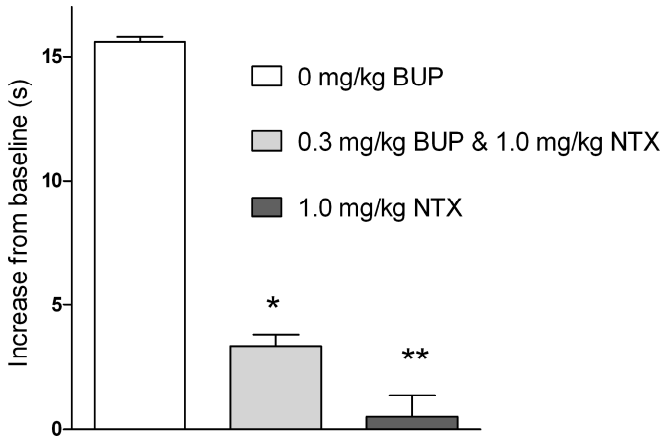
Increase from baseline (s)
15
10
5
0
0 mg/kg BUP
0.3 mg/kg BUP & 1.0 mg/kg NTX
1.0 mg/kg NTX
*
**

FIGURE 3

	Cocaine control cohort	Morphine control cohort	Cocaine treated cohort	Morphine treated cohort
At start	20	24	16	16
Excluded for preference	0	2	2	1
Excluded for insufficient conditioning	8	8	5	7
Time taken to reach extinction	1 week	1 week or 12 days	1 week	1 week

TABLE 1

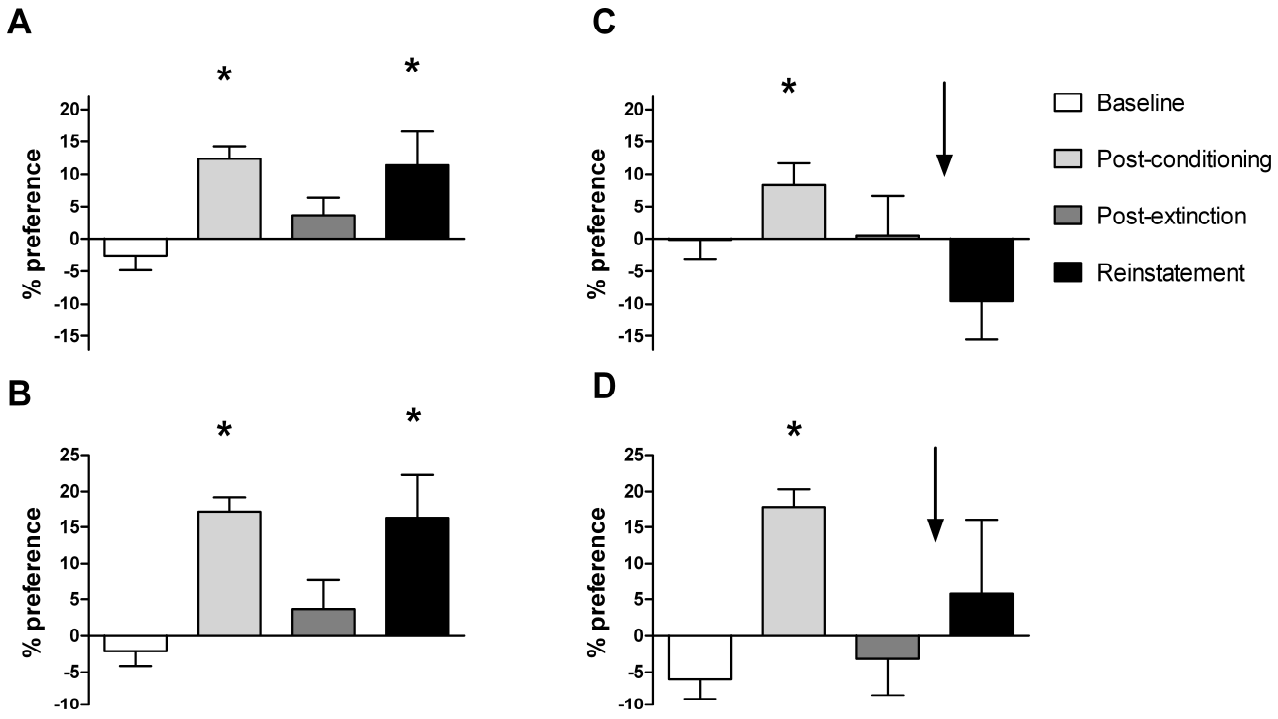
A
B
C
D
% preference
Baseline
Post-conditioning
Post-extinction
Reinstatement

FIGURE 4

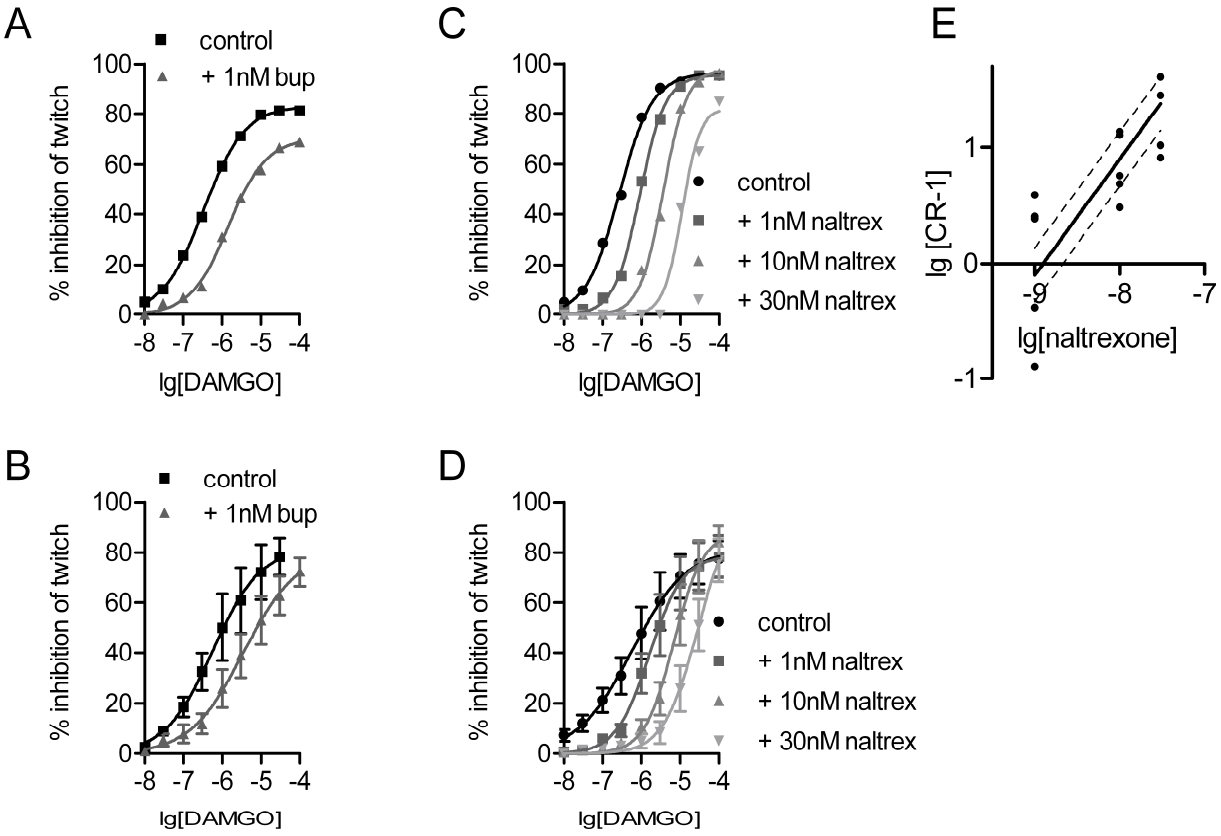
A
control
+ 1nM bup
% inhibition of twitch
lg[DAMGO]
B
control
+ 1nM bup
% inhibition of twitch
lg[DAMGO]
C
control
+ 1nM naltrex
+ 10nM naltrex
+ 30nM naltrex
% inhibition of twitch
lg[DAMGO]
D
control
+ 1nM naltrex
+ 10nM naltrex
+ 30nM naltrex
% inhibition of twitch
lg[DAMGO]
E
lg [CR-1]
lg[naltrexone]

FIGURE 5

	Buprenorphine 0.3 mg/kg		Naltrexone 1.0 mg/kg	
	Observed concentration (nM)	Predicted occupancy of mu receptor (%)	Observed concentration (nM)	Predicted occupancy of mu receptor (%)
Plasma	56 ± 4	38	285 ± 19	62
Brain	83 ± 14	45	313 ± 19	55

TABLE 2