



Citation for published version:

Almatroudi, A, Husbands, SM, Bailey, CP & Bailey, SJ 2015, 'Combined administration of buprenorphine and naltrexone produces antidepressant-like effects in mice', *Journal of Psychopharmacology*, vol. 29, no. 7, pp. 812-821. <https://doi.org/10.1177/0269881115586937>

DOI:

[10.1177/0269881115586937](https://doi.org/10.1177/0269881115586937)

Publication date:

2015

Document Version

Peer reviewed version

[Link to publication](#)

Almatroudi, Abdulrahman ; Husbands, Stephen M ; Bailey, Christopher P ; Bailey, Sarah J. / Combined administration of buprenorphine and naltrexone produces antidepressant-like effects in mice. In: *Journal of Psychopharmacology*. 2015 ; Vol. 29, No. 7. pp. 812-821. (C) The Author(s) 2015. Reprinted by permission of SAGE Publications.

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Combined administration of buprenorphine and naltrexone produces antidepressant-like effects in mice.

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KEY WORDS: depression, anxiety, kappa opioid receptor, kappa antagonist, mu agonist

SOURCES OF SUPPORT: This research is funded by the Government of Saudi Arabia through a PhD scholarship (AA), the Royal Society (SJB) and NIDA DA07315 (SMH) .

ABSTRACT

Opiates have been used historically for the treatment of depression. Renewed interest in the use of opiates as antidepressants has focussed on the development of kappa opioid receptor (κ -receptor) antagonists. Buprenorphine acts as a partial μ -opioid receptor agonist and a κ -receptor antagonist. By combining buprenorphine with the opioid antagonist naltrexone, the activation of μ -opioid receptors would be reduced and the κ -antagonist properties enhanced. We have established that a combination dose of buprenorphine (1mg/kg) with naltrexone (1mg/kg) functions as a short-acting κ -antagonist in the mouse tail withdrawal test. Furthermore, this dose combination is neither rewarding nor aversive in the conditioned place preference paradigm and is without significant locomotor effects. We have shown for the first time that systemic co-administration of buprenorphine (1mg/kg) with naltrexone (1mg/kg) in CD-1 mice produced significant antidepressant-like responses in behaviours in both the forced swim test and novelty induced hypophagia task. Behaviours in the elevated plus maze and light dark box were not significantly altered by treatment with buprenorphine alone, or in combination with naltrexone. We propose that the combination of buprenorphine with naltrexone represents a novel, and potentially a readily translatable approach, to the treatment of depression.

Introduction

Opioid receptors are increasingly recognized to play a role in the regulation of mood and emotional behaviours (Lutz and Kieffer, 2013). Among the opioid receptors, kappa opioid receptors (κ -receptor) have recently been a focus for antidepressant and anxiolytic drug development (Carroll and Carlezon, 2013; Van't Veer and Carlezon, 2013). Dynorphins are endogenous neuropeptides that activate κ -receptors. Both dynorphins and κ -receptors are highly expressed in brain regions that mediate stress responses, cognitive and reward behaviours such as the amygdala, hypothalamus, locus coeruleus, hippocampus, ventral tegmental area and nucleus accumbens in human and rodent brains (Kitchen et al., 1997; Mansour et al., 1994). Dynorphin, released during stress exposure, or administration of κ -agonists induces prodepressive-like behaviour in rodents including increased immobility in the forced swim test (Carlezon et al., 2006; McLaughlin et al., 2003; Shirayama et al., 2004). In contrast, κ -receptor gene deletion or prodynorphin gene disruption has the ability to block stress induced prodepressive-like effects (McLaughlin et al., 2003; Wittmann et al., 2009). It has also been demonstrated that selective high affinity κ -receptor antagonists, such as norbinaltorphimine (norBNI), effectively reduce stress-induced prodepressive-like behaviours and have antidepressant-like and anxiolytic-like effects in rodents (Knoll et al., 2007; Mague et al., 2003; McLaughlin et al., 2003). Taken together these data suggest that κ -receptor antagonists may be effective in the treatment of comorbid depression and anxiety.

The existing high affinity selective κ -antagonists, such as norBNI and 5'-guanidinonaltrindole, have two distinct pharmacological properties; slow onset of antagonist activity and very long lasting effects in vivo (Beguin and Cohen, 2009;

Carroll and Carlezon, 2013). For example, one injection of norBNI has peak κ -antagonist effects starting at about 24 h post-administration, continuing at high levels for 7–10 days and returning to control levels after 3–4 weeks or persisting for months (Endoh et al., 1992; Horan et al., 1992). This limits in vivo behavioural testing and potentially clinical trials if the blockade of κ -receptors may not be easily reversed. A variety of mechanisms may account for the unusual long duration of action of selective κ -antagonists including high lipophilicity leading to slow clearance from the brain, production of metabolites that are active at the receptors, resistance to metabolism which increases bioavailability or ligand-directed signalling producing a c-Jun N terminal kinase (JNK) mediated inactivation of the κ -receptor (Beguin and Cohen, 2009; Bruchas and Chavkin, 2010). To overcome this issue, a number of selective shorter acting κ -receptor antagonists have recently been synthesised and characterised but have not yet reached the clinic (Casal-Dominguez et al., 2014; Eans et al., 2013; Peters et al., 2011; Ross et al., 2012; Verhoest et al., 2011).

We have explored an alternative approach, using two drugs that are already licensed for other therapeutic indications. Buprenorphine is a semi-synthetic opioid with a unique complex pharmacology. Buprenorphine acts as a partial μ -receptor agonist and a κ -receptor antagonist with additional nociception/orphanin FQ receptor (NOP-receptor, also known as ORL1) partial agonist activity (Huang et al., 2001; Lutfy and Cowan, 2004). Clinically, buprenorphine is used as a potent analgesic and as an alternative to methadone in the treatment of opioid addiction (Maremmani and Gerra, 2010). In addition, buprenorphine has been shown to be effective in a small cohort of treatment-resistant depressed patients with clinical improvement evident within 1 week of treatment (Bodkin et al., 1995). A more recent study, in a small cohort of

older treatment-resistant depressed adults, also suggests clinically significant improvements within 3 weeks of treatment (Karp et al., 2014). Recently, buprenorphine has also been shown to have antidepressant- and anxiolytic-like activity in mice (Falcon et al., 2014). However, treatment with μ -agonists carries a risk of abuse liability and dependence. Naltrexone, is a relatively non-selective opioid receptor antagonist, with a higher affinity for μ - than κ -opioid receptors. In the UK it is licensed as an abstinence promoter for the treatment of alcohol addiction (Rosner et al., 2010). Combining naltrexone with buprenorphine could reduce the potential abuse liability of buprenorphine activating μ -receptors, while enhancing κ -receptor antagonist actions. Buprenorphine-naltrexone has proved safe and effective in treating drug dependence as it improves the dysphoria associated with drug withdrawal (Gerra et al., 2006). Also, it has recently been reported that a combination of buprenorphine with a μ -receptor antagonist samidorphan has antidepressant effects in major depressive disorder patients (Ehrich et al., 2015). Taken together these data suggest that buprenorphine in combination with naltrexone may have clinical utility as an antidepressant.

The aim of this study was to identify doses of buprenorphine and naltrexone that when co-administered in mice, produces a functional, short-acting κ -antagonist. Subsequently, we have tested the dose combination to ensure that it is not sedating, nor rewarding, nor aversive. Finally, we have evaluated whether combination treatment with buprenorphine and naltrexone has antidepressant- and anxiolytic-like potential in mice.

Methods and Materials

Drugs

Diazepam and U50,488 were purchased from Sigma (Dorset, UK). Fluoxetine and naltrexone were supplied by Abcam Biochemicals (Cambridge, UK). Clocinnamox (CCAM) and norBNI were obtained from Tocris Bioscience (Bristol, UK). Buprenorphine was purchased from MacFarlan Smith (Edinburgh, UK). For in vivo experiments, all drugs were dissolved in 0.9% w/v saline (Hameln Pharmaceuticals, Gloucester, UK) and injected via the intraperitoneal route at a volume of 10ml/kg, except for CCAM which was injected at a volume of 20ml/kg.

Animals

Adult (8–10 weeks, 25–43 g) male CD-1 mice, bred in-house at the University of Bath for more than 10 years (originally from Charles River Crl:CD1(ICR)), were housed in groups of 4 to 5. In the homecages (30 x 16 x 14 cm), mice were provided with wood shavings and nesting material with ad libitum access to food and water. Mice were maintained on a 12:12 hours light–dark cycle (lights on 07:00 h; lights off 19:00 h) at $20 \pm 2^\circ\text{C}$. All experiments were performed in accordance with the UK Home Office guidelines and the Animals (Scientific Procedures) Act 1986. For all behavioural tasks animals were habituated to the behavioural room for one hour prior to the experiment beginning. Separate groups of animals, $n=5-10$ per treatment group, were used for each behavioural task. All behavioural experiments were performed between 9:00-16:00 h and mice were acclimatized to the behavioural room for 1h prior to starting.

Warm water tail-withdrawal test

The mice were scruffed and held in a vertical position, and the distal third of the tail lowered into a water bath maintained at 52°C. The latency for the mouse to withdraw the tail was recorded. A 15-second cut-off was imposed to avoid tissue damage. Antinociception was calculated as percentage maximum possible effect (%MPE) = $(\text{test latency} - \text{control latency}) / (15 \text{ s} - \text{control latency}) \times 100$. To determine the time course of the antinociceptive effects of buprenorphine (1 mg/kg) and the κ -agonist U50,488 (10 mg/kg), baseline latencies were determined 30 minute before injecting these drugs at time zero. To determine effective μ and κ -antagonist doses (Figure 1 A,B,C,D), naltrexone (0.3, 1 and 3 mg/kg) was injected 10 minutes prior to buprenorphine or U50,488 administration. Tail-withdrawal responses were measured at 30, 60, 120, 240 minute and 24 hours post-injection.

To examine the duration of the κ -antagonist actions (Figure 1E), tail-withdrawal latency was measured at 1, 8, 24 and 48 h post-administration of antagonist, naltrexone (1 mg/kg) alone, or in combination with buprenorphine (1 mg/kg). In these experiments, naltrexone or saline was injected 10 mins before time zero. Buprenorphine or saline was injected at time zero. U50,488 or saline was injected 30 minute before taking a measurement to assess the extent of κ -receptor blockade. In experiments with the irreversible, selective μ -antagonist CCAM (Broadbear et al., 2000), CCAM was administered 24 h before treatment with buprenorphine/naltrexone (1 mg/kg) combination. To counteract any possible confounding effects of injection induced stress, in all experiments, animals received 0.9% w/v saline injections so that the total number of injections an individual mouse received, whether in control or in drug treated groups, was equivalent.

Conditioned place preference (CPP)

Place preference conditioning was conducted in a CPP chamber with an auto monitoring system (Ethovision XT version 8.0). The apparatus (UGO Basile) consisted of a box with two compartments (16 x 15 cm / compartment) joined by a removable partition that allowed mice to freely explore or be restricted to a particular compartment. The two compartments differed in appearance and texture; one compartment had black walls and a grey floor with round 2mm holes whilst the other compartment had walls with vertical black and white stripes and a grey floor with 4 x 4 mm square holes. Experiments were performed under dim light (approximately 15 lux). During all test sessions, the time each mouse spent in each compartment was recorded using tracking software.

Mice were randomly assigned to treatment groups and the pairing was counterbalanced (i.e. within each treatment group equal numbers of mice were always drug-paired to each compartment type). On days 1 and 2 mice were habituated to the entire chamber for 15 min (one session /day). On days 3-8 mice were conditioned (30 min) to one of the two compartments and daily sessions alternated between drug treatment and saline (in all treatment groups mice received both drug and saline). Three drug treatment groups (n=8 per group) were used and mice received buprenorphine (1mg/kg) administered 10 min after 0.9 % saline or naltrexone (1 mg/kg) or naltrexone (3 mg/kg). After buprenorphine injection the mice were transferred directly to the place preference box and at the end mice were returned to their home cage. Chamber floors and trays were removed and cleaned with ethanol 70 % and left for 5 minutes for ethanol to evaporate before the next trial. On day 9, mice were not injected with saline or drugs. In a free-to-explore test,

lasting 15 minutes, mice had free access to both compartments and their preference was determined by recording the time spent in the drug-paired chamber.

Locomotor activity

Locomotor activity was assessed in an open-field test . Testing was performed to establish the potential sedative effects of buprenorphine (1mg/kg) alone or in combination with naltrexone (0.3, 1 mg, and 3 mg/kg). Naltrexone was injected 10 minute before buprenorphine. One hour post-administration, mice were placed singly in an open field (72 x 72 cm) for 10 minute under low light conditions (30 lux). Total activity was recorded by photobeam breaks using Motor Monitor software (Campden Instruments).

Forced swim test

Mice were individually placed inside a glass beaker (height 44 cm x diameter 22 cm) filled with water at a depth of 30 cm, at 25±2°C and behaviour recorded (Sony DCR-SR52) for 6 min (Casal-Dominguez et al., 2013). Mice were removed, dried and returned to their home cages. Mice were scored, blind for treatment, for three measures: swimming, immobility, climbing and the time spent engaged in these behaviours in the last 4 minutes of the test reported. Drug treatments were saline-injected controls, buprenorphine (1mg/kg) alone, naltrexone (1mg/kg) alone, buprenorphine/naltrexone (1mg/kg) combination and the selective serotonin reuptake inhibitor (SSRI) fluoxetine (20 mg/kg). All drugs were administered 1 hour prior to testing (naltrexone 10 min before buprenorphine).

Novelty-induced hypophagia

Mice were individually housed for 3 days before training began. Training consisted of 3 consecutive days in which mice received concentrated milk (1:3; sweetened condensed milk: water) for 30 minute in their home cage and lighting levels were set to 20 lux (Dulawa and Hen, 2005). On day 4 mice underwent home cage testing. On day 5, novel cage testing was conducted by placing the mouse in a clean cage of the same dimensions as their home cage, but with no bedding or shavings and under bright lighting (300 lux, Fig 5A; 500 lux Fig 5B). The latency to drink was recorded during a 30-min test period in both the home and novel cage environments. In the first experiment (Figure 5A), mice received buprenorphine and naltrexone alone, or combined, or the SSRI fluoxetine (20 mg/kg) 1 h prior to testing (naltrexone 10 min before buprenorphine). Mice were also administered the κ -antagonist norBNI (10mg/kg; 24-48 h prior to testing, after training on day 3). In the second experiment (Figure 5B), the lighting was increased to make the novel cage more aversive and the ability of the irreversible μ -antagonist CCAM (3 mg/kg) to block the effects of buprenorphine and naltrexone was investigated by administration after training on day 3.

Elevated plus maze (EPM)

Mice were placed in the centre of an EPM (EPM2000 Mouse Plus Maze, Campden Instruments) facing an open arm and behaviour was recorded for 5 min (Casal-Dominguez et al., 2013). The time spent in, and entries into, the open and closed arms, and total ambulation were recorded via infrared photobeams and analyzed with Motor Monitor™ software (Campden Instruments). Illumination was 150 lux in the open arms and <1 lux in the closed arms. Mice (n=10/group) were treated with saline, buprenorphine (1 mg/kg) alone, naltrexone (1mg/kg) alone, buprenorphine/naltrexone

(1mg/kg) combination and diazepam (2mg /kg), a positive control, 1h prior to testing (naltrexone 10 min before buprenorphine).

Light Dark Box (LDB)

Mice were placed at the centre of the lit compartment (400 lux), facing the dark compartment and allowed free access between compartments for 10 min (Openfield SmartFrame, Campden Instruments) (Casal-Dominguez et al., 2013). The time spent in, and number of entries into, the lit compartment, and the distance travelled in the LDB were recorded via beam-breaks using Motor Monitor™ software (Campden Instruments). Mice (n=10/group) were treated with saline, buprenorphine (1 mg/kg) alone, naltrexone (1mg/kg) alone, buprenorphine/naltrexone (1mg/kg) combination and diazepam (2mg /kg) 1h prior to testing (naltrexone 10 min before buprenorphine).

Statistical analysis

All behavioural scoring was performed blind to treatment and data were analyzed using two-way repeated measures mixed model analysis or single measures one-way ANOVA followed by Unadjusted Least Significant Difference (ULSD) post hoc test (Invivostat 2.3). Only planned pairwise tests were carried out and *p* values adjusted for multiple comparisons with Benjamin-Hochberg correction. Values are reported as mean ± standard error of the mean (SEM) for each treatment group.

Results

Establishing a combination dose of buprenorphine/naltrexone that is a functional kappa opioid receptor antagonist

We used CD-1 mice for these studies as we have previously shown that this strain responds to the anxiolytic and antidepressant effects of a range of κ -antagonist compounds (Casal-Dominguez et al., 2013). We first established doses of buprenorphine that would provide robust antinociception via activation of μ -opioid receptors in the warm water tail withdrawal assay. The antinociceptive activity of buprenorphine has been reported to have an inverted U-shaped dose response curve, with reduced antinociception at high doses (Lutfy and Cowan, 2004). Our data demonstrated that in CD1 mice 1 mg/kg buprenorphine produced a significant antinociceptive effect that was not enhanced at 3mg/kg and not evident at 0.3 mg/kg (Supplementary Figure 1). Furthermore, over this dose range, buprenorphine was without significant effects on locomotor activity (Supplementary Figure 2).

We next established doses of naltrexone, a relatively non-selective opioid receptor antagonist (Giordano et al., 1990), that would block the partial μ -receptor agonist activity of buprenorphine using the warm water tail-withdrawal test (Figure 1A,B, n=5 per group). Two-way repeated measures mixed model analysis revealed a significant interaction of Treatment * Time ($F_{(24,120)} = 2.46, p < 0.001$). Buprenorphine (1mg/kg) produced a significant antinociceptive effect that peaked at 60 min post-administration ($p < 0.001$, compared to saline injected controls, figure 1B) returning to baseline after 240 min. Pre-treatment with naltrexone 1mg and 3mg/kg, but not 0.3mg/kg, significantly blocked the buprenorphine-induced antinociception at 30 min ($p < 0.01$) and 60 min ($p < 0.001$). To determine the κ -antagonist properties of naltrexone, the κ -agonist U50,488 (10mg/kg) was used (Figure 1C,D, n=5 per group).

In this experiment there was a significant Treatment * Time interaction ($F_{(24,114)} = 2.12$, $p < 0.004$). U50,488 produced a significant antinociceptive effect 30 min post-administration ($p < 0.01$) that persisted for more than 240 min ($p < 0.05$ compared to saline controls). Pretreatment with naltrexone 1mg and 3mg/kg, but not 0.3 mg/kg, significantly blocked the U50, 488-induced antinociception (all p 's < 0.01 compared to U50,488 alone).

To determine whether naltrexone alone or buprenorphine/naltrexone combination has long acting κ -antagonist effects, their ability to block U50,488-induced antinociception was tested at 1, 8, 24 and 48 h post-administration of antagonist (Figure 1E, $n=5$ per group). Two-way repeated measures mixed model analysis revealed that there was a significant interaction of Treatment * Time ($F_{(12,64)} = 12.25$, $p < 0.001$). At 1 hour post-administration U50,488 produced a pronounced antinociceptive effect that was significantly reduced by naltrexone alone, or in combination with buprenorphine, 1 h post-administration (all p 's < 0.001 , compared to U50,488 alone). The κ -receptor blockade by naltrexone and by combination buprenorphine/naltrexone was not evident at 24 and 48h post-administration of antagonist (Figure 1E). At 8h post-administration of naltrexone, its ability to block U50,488 induced antinociception was reduced, compared to 1h. Interestingly, at 8 hr post administration of buprenorphine/naltrexone, U50,488 induced an apparently potentiated antinociceptive effect ($p < 0.05$ compared to U50,488 alone). In a separate experiment, the irreversible μ -antagonist CCAM (3mg/kg), administered 24h before this tail withdrawal assay was repeated, blocked this apparent potentiation of U50,488 effects by buprenorphine/naltrexone at 8h, suggesting that it is a result of μ -receptor activation by buprenorphine or its metabolites (Figure 1F). Overall, these data show

that at 1 and 3mg/kg, naltrexone is an effective μ - and κ -antagonist, with κ -antagonist effects that are evident at 1 h post administration, reversing at 8h and completely reversed at 24 h. The combination of buprenorphine (1mg/kg) with naltrexone (1 mg/kg) also produces a functional κ -antagonist with a rapid onset and a duration of action < 24 h.

Effects of buprenorphine and naltrexone on locomotor activity

To assess the locomotor effects of the ligands used in this study, behaviour in the open-field test was investigated (Figure 2). Buprenorphine alone (1 mg/kg) or in combination with naltrexone (0.3, 1, and 3 mg/kg), had no significant effects on total locomotion compared to saline treated controls ($F_{(4,20)} = 0.62$, $p = 0.657$). Naltrexone (1mg/kg) alone had no significant locomotor effects (see Supplementary Figure 2).

Establishing the rewarding properties of combination buprenorphine/naltrexone in conditioned place preference (CPP) task

Mice receiving 1 mg/kg buprenorphine exhibited significant conditioned place preference, evident as a significantly increased time spent in the drug-paired compartment of the CPP chamber, compared to pre-conditioning (Figure 3) (pre-conditioning: 451.6 ± 25.2 s, post-conditioning: 587.1 ± 73.0 s; $n = 8$, $p = 0.05$). However, co-administration of 1 and 3 mg/kg naltrexone completely blocked the conditioned place preference elicited by buprenorphine. While not significant, there was a trend for 3mg/kg naltrexone to increase the time spent in the saline-paired compartment compared with preconditioning, suggesting that naltrexone at this dose may be

eliciting an aversive response. Therefore, in subsequent behavioural experiments, the combination of 1mg/kg buprenorphine and 1mg/kg naltrexone was used.

Effects of combination buprenorphine/naltrexone on depression- and anxiety-related behaviours

The effects of buprenorphine (1mg/kg) and naltrexone (1 mg/kg), alone or in combination, were compared with the SSRI fluoxetine (20mg/kg) in the forced swim test (Figure 4A, n=10 per group). One-way ANOVA revealed a significant effect of Treatment on the time spent swimming ($F_{(4, 45)} = 6.88, p < 0.001$) and immobile ($F_{(4, 45)} = 6.97, p < 0.001$). Post hoc comparisons to saline treated controls revealed that all drug treated groups increased the time spent swimming and decreased the time spent immobile during the last 4 minute of the test (all p 's < 0.001). Interestingly, immobility times for buprenorphine (1mg/kg) and naltrexone (1mg/kg) administered alone were not significantly different from the combination treatment.

We further investigated whether the antidepressant-like effects of buprenorphine in the forced swim test were related to its partial μ -agonist activity (Figure 4B). The irreversible μ -antagonist CCAM (3mg/kg) was administered 24 h before buprenorphine or saline were injected and activity assessed 1 h later in the FST. One-way ANOVA revealed a significant effect of Treatment on the time spent swimming ($F_{(4, 40)} = 8.84, p < 0.001$) and immobile ($F_{(4,40)} = 7.77, p < 0.001$). Buprenorphine alone, or in combination with CCAM, produced a significant increase in swimming, and a decrease in immobility, compared with saline ($p < 0.01$). CCAM alone produced no significant effects on behaviour in the forced swim test. These data

suggest that the antidepressant-like effects of buprenorphine alone were not mediated by effects at the μ -opioid receptor.

Behaviour of mice administered buprenorphine (1mg/kg) and naltrexone (1 mg/kg), alone or in combination, were compared with the SSRI fluoxetine (20mg/kg) and the κ -antagonist norBNI (10mg/kg) in the novelty-induced hypophagia task (Figure 5A, n= 10 per group). Two-way repeated measures mixed model analysis of the latency to drink times revealed significant main effects of Treatment ($F_{(5, 54)} = 11.64$, $p < 0.001$) and a significant Treatment * Environment interaction ($F_{(5, 54)} = 10.78$, $p < 0.001$). Post hoc comparisons of behaviours in the novel cage showed that naltrexone alone, or in combination with buprenorphine, significantly reduced the latency to drink milk in the novel cage ($p < 0.05$, compared to saline controls). In addition, the novel cage was aversive as demonstrated by the significant increase in the latency to drink in saline treated mice in the novel cage (Mean latency value = 7.52 ± 0.86 min) compared with the home cage environment (Mean latency value = 0.53 ± 0.18 min, $p < 0.001$). Moreover, the SSRI fluoxetine (administered acutely 30 min before testing) and the κ -antagonist norBNI (administered at the end of training on day 3) also significantly reduced the latency to drink in the novel cage ($p < 0.01$, compared to saline).

Interestingly, buprenorphine alone significantly increased the time to drink milk in the home cage ($p < 0.001$) as compared to saline and all drug treated groups. However, there was no significant difference between buprenorphine and saline

treated controls in the novel cage ($p= 0.458$). In a subsequent experiment (Figure 5B), we increased the lighting in the novel cage to enhance its aversive effects (from 300 lux to 500 lux). We also tested the ability of the irreversible μ -antagonist CCAM (3mg/kg) to block the effects of buprenorphine and naltrexone in the NIH test (Figure 5B, $n=9$ per group). In CCAM treated mice, CCAM was administered at the end of training on day 3. Two-way repeated measures mixed model analysis revealed significant main effects of Treatment ($F_{(6, 56)} =9.17$ $p<0.001$) and an interaction between Treatment * Environment ($F_{(6, 56)} =26.39$, $p<0.001$). Post hoc comparisons revealed that, under these conditions, buprenorphine alone significantly reduced the latency to drink in the novel cage ($p<0.05$ compared to saline control). CCAM blocked the effects of buprenorphine in the home cage to increase the latency to drink ($p<0.001$) indicating that this effect was mediated by the μ -receptor. In both the home and novel cages, CCAM alone was without significant effect on the latency to drink, compared to saline controls. Furthermore, CCAM did not block the effects of buprenorphine or naltrexone in the novel cage, indicating that these effects on latency to drink in the novel cage are not μ -receptor mediated.

Analysis of behaviours in the EPM, using one-way ANOVA, revealed significant effects of Treatment on the time spent in ($F_{(4, 45)} =3.32$, $p< 0.05$) and number of entries into ($F_{(4, 45)} =4.42$, $p<0.01$) the open arms (Figure 6 A,B,C, $n= 10$ per group). Within treatment comparisons to saline treated controls revealed that only the benzodiazepine diazepam (2 mg/kg) significantly increased these parameters ($p <0.05$). Neither buprenorphine nor naltrexone, alone or in combination, significantly

affected behaviours in the EPM. Total ambulation in the EPM was not affected by drug treatment ($F_{(4, 45)}=0.95$ $p=0.441$) showing an absence of any locomotor effects.

Similarly, in a separate group of mice, in the LDB there were no significant effects of treatment with buprenorphine and naltrexone, alone or in combination (Figure 6 D,E,F, $n= 18$ per group). One-way ANOVA revealed significant main effects of Treatment on the time spent in the light ($F_{(4, 85)}=3.02$, $p< 0.05$) and dark ($F_{(4, 85)}=2.81$, $p< 0.05$) compartment. Within treatment comparisons to saline controls showed that only diazepam (2mg/kg) significantly increased the total time spent in the light compartment ($p<0.05$). As with the EPM, total ambulation in the LDB was not significantly affected by drug treatment ($F_{(4, 85)}= 2.0$, $p= 0.102$) confirming that locomotor effects were not a confound in these experiments.

DISCUSSION

Here, we have shown for the first time that systemic co-administration of buprenorphine (1mg/kg) with naltrexone (1mg/kg) in CD-1 mice produced an antidepressant-like response in behaviours in both the forced swim test and novelty induced hypophagia task, suggesting that this drug combination has potential as an antidepressant. Interestingly, the combination of buprenorphine with naltrexone was without significant effect on anxiety-related behaviours in the EPM and LDB. We have established that this combination dose of buprenorphine (1mg/kg) with naltrexone (1mg/kg) functions as a short-acting κ -antagonist in the tail withdrawal test. Furthermore, this dose combination is neither rewarding nor aversive in the CPP paradigm and is without significant locomotor effects.

The antidepressant potential of buprenorphine and naltrexone arises from studies of this combination as a treatment for opioid dependence (Gerra et al., 2006; Rothman et al., 2000). Naltrexone is well established as a treatment for opioid and alcohol dependence but patient compliance is low. Possible reasons for low adherence include the aversive side effects of naltrexone treatment and the fact that naltrexone has little effect on anhedonia symptoms associated with opioid withdrawal (Bouza et al., 2004; Gerra et al., 2006). The combination of naltrexone (50 mg oral dose) plus buprenorphine (4mg sublingual) improves mood and reduces the intensity of dysphoria, leading to improved retention of addicts in treatment (Gerra et al., 2006; Rothman et al., 2000). These authors have suggested that this drug combination produces κ -antagonism which improves mood states. Our data with the irreversible μ -antagonist CCAM supports this idea. In both the forced swim test and novelty-induced

hypophagia task, blockade of μ -receptors did not affect the antidepressant-like response produced by treatment with combination buprenorphine/naltrexone indicating that these effects are mediated via κ -opioid receptors, rather than μ -receptors.

Buprenorphine, a partial μ -receptor agonist and a κ -antagonist, has itself previously been shown to have antidepressant effects in depressed patients (Emrich et al., 1982) and in treatment resistant depressed patients (Bodkin et al., 1995; Karp et al., 2014). A number of preclinical trials have also demonstrated antidepressant effects of μ -receptor agonist activation. Endogenous enkephalins and endorphins reduced immobility and increased activity of swimming in rats (Kastin et al., 1978). In the mouse tail suspension test endomorphins (Fichna et al., 2007), morphine, codeine and other agonists reduced the time spent immobile (Berrocoso et al., 2013). More recently, low doses of buprenorphine (0.25 mg/kg and 0.5 mg/kg) have been shown to have antidepressant-like effects in the NIH and FST in C57BL/6 J mice (Falcon et al., 2014).

However, buprenorphine's partial μ -agonist activity carries a risk of abuse liability. While buprenorphine is not usually preferred as a primary drug of abuse, the non-therapeutic use of buprenorphine has recently been reported to be rising among drug users as it serves as a substitute for other drugs of abuse (Cicero et al., 2014). The abuse potential would make buprenorphine alone, a less attractive antidepressant therapy. However, combining buprenorphine with an opioid antagonist would reduce the abuse liability. This is the approach that Alkermes have

taken in a recent placebo controlled study of the effects of buprenorphine in combination with a potent μ -opioid antagonist samidorphan (Ehrich et al., 2015). Following the calibration of samidorphan's ability to block buprenorphine-induced effects (e.g. dose-dependent reductions in post-administration miosis) in opioid-experienced adults, the study evaluated the effects of buprenorphine (2,4 and 8 mg), in 8:1 and 1:1 dose ratios with samidorphan, as adjunctive therapies in a small cohort of adult subjects with major depressive disorder. Patients had a current episode of depression and experienced inadequate response to antidepressant treatment. After 7 days of once daily buprenorphine/samidorphan, at a 1:1 ratio, depressed patients exhibited a statistically significant improvement in HAM-D17 total score versus placebo, with an effect size of 1.49 (Ehrich et al., 2015). While this is a short duration study in a small cohort of patients, it does demonstrate that the approach of combining buprenorphine with an opioid antagonist has clinical therapeutic potential.

Naltrexone is often reported to have aversive effects. However, we have shown that naltrexone administered alone produces a significant antidepressant-like effect in the forced swim test and novelty induced hypophagia paradigm. Naltrexone is a relatively non-selective opioid receptor antagonist, with a higher affinity for μ - rather than κ -receptors (Giordano et al., 1990). Hence, we anticipated that naltrexone would reduce buprenorphine's activation of μ -receptors while potentially enhancing its κ -receptor antagonist actions. We have previously shown that mixed μ/κ -receptor antagonists produce both antidepressant and anxiolytic effects in CD-1 mice (Casal-Dominguez et al., 2013). In overweight healthy volunteers, a daily 200 mg dose of naltrexone was found to have no effect on mood symptoms over a 10

week period (Malcolm et al., 1987). However, in opioid dependent patients, with a high baseline affective burden, depot naltrexone treatment produced a significant improvement in depression scores (Mysels et al., 2011). Perhaps there is therapeutic potential for exploiting the mixed, relatively non-selective opioid receptor antagonists as antidepressant treatments, especially in patients with comorbid substance misuse and mood disorders (Pettinati et al., 2013).

One important caveat with these findings is that they are based on mouse behavioural paradigms. The forced swim test is not a model of depression but is a well-validated and well-established behavioural task for assessing acute antidepressant efficacy (Cryan et al., 2002; Petit-Demouliere et al., 2005). Interestingly, in the forced swim test, antidepressants that target the serotonergic system increase swimming behaviour, whereas those that target noradrenergic systems increase climbing behaviour, thereby decreasing immobility (Detke et al., 1995). In our experiments, buprenorphine and naltrexone treatment decreased immobility with a concomitant increase in swimming behaviour, without an effect on climbing behaviour. This may indicate that serotonergic pathways are implicated in the opioid mediated antidepressant effects seen here, as has been suggested by others (Bruchas et al., 2011). However, it has been argued that the forced swim test has limited predictive validity and that behavioural paradigms responding to chronic antidepressant treatments have greater validity (Mitchell and Redfern, 2005). The novelty-induced hypophagia task is a procedure that has been developed to assess anxiety-related behaviours but has been shown to be sensitive to the chronic anxiolytic effects of antidepressants in rodents (Dulawa and Hen, 2005). In our study, the combination of buprenorphine and naltrexone has been shown to have

antidepressant-like effects in both the forced swim test and the novelty-induced hypophagia test in adult male CD-1 mice. Further studies are required to assess whether buprenorphine/naltrexone has any utility in animal models of depressive symptoms such as the Flinders Sensitive Line rat or the chronic unpredictable mild stress model (Neumann et al., 2011; Papp et al., 1991).

Anxiety-related behaviours have been reported to be regulated by the dynorphin- κ -receptor system. For example, dynorphin induced significant anxiogenic-like effects in mice in the LDB and EPM (Narita et al., 2005), while κ -antagonists produced acute and persistent anxiolytic-like effects (Knoll et al., 2007). Interestingly, buprenorphine, over a similar dose range used here (0.3, 1 and 3 mg/kg) has been reported to show anxiogenic effects in NMRI mice in the LDB test (Lelong-Boulouard et al., 2006). However, in our experiments there was no evidence of buprenorphine's reported anxiogenic effects. On the contrary, in the LDB, there was an apparent trend for buprenorphine (and buprenorphine/naltrexone combination) to increase the time spent in the lit compartment, almost double in comparison with saline controls, although these results did not achieve statistical significance. The absence of a robust anxiolytic-like response in the EPM and LDB was surprising but this may be because the mice were not sufficiently stressed in these paradigms to activate dynorphin release and alter anxiety behaviours (McLaughlin et al., 2006; Shirayama et al., 2004; Wittmann et al., 2009). Hyponeophagia, such as in tested in the novelty induced hypophagia task, is an anxiety-related measure that is sensitive to the effects of a wide range of pharmacological manipulations including benzodiazepines and SSRIs (Dulawa and Hen, 2005). Both acute and repeated administration of low dose buprenorphine has recently been shown to reduce the latency to approach food in a

novelty induced hypophagia task (Falcon et al., 2014). The novelty induced hypophagia test is a conflict-based anxiety test where the aversive novel cage environment suppresses the approach to a highly palatable food. The demonstration of an effect of combination buprenorphine/naltrexone in the novelty induced hypophagia task, but not in EPM and LDB, supports the potential of κ -antagonists in stress-related tasks (Cryan and Sweeney, 2011).

We have shown that the combination of buprenorphine (1mg/kg) with naltrexone (1mg/kg) administered intraperitoneally in CD-1 mice has antidepressant potential. Both drugs are licensed currently for other indications so may be attractive to translate to the clinic. However, naltrexone is administered orally and buprenorphine sublingually, so achieving the correct dose combination to achieve an antidepressant effect may not be trivial. Cordery et al. (2014) have suggested that the ideal buprenorphine: naltrexone plasma concentration ratio is around 1:5 for anti-addiction treatment. Further, they suggested that higher doses of both buprenorphine and naltrexone than those used by Rothman et al. (2000) and Gerra et al. (2006) for treatment of opioid dependence (buprenorphine 4 mg daily/ naltrexone 50 mg daily) may be even more effective clinically as the combination would result in greater receptor occupancies. In preclinical studies, lower doses of buprenorphine at 0.25 and 0.5 mg/kg have been shown to have antidepressant-like effects in mice (Falcon et al., 2014), in comparison to 1mg/kg used in this study which also produced antidepressant-like effects. Interestingly, clinically significant effects of buprenorphine have been observed at low doses with the titrated dose ranging from 0.15-1.8 mg/d or 0.2-1.6 mg/d (Bodkin et al., 1995; Karp et al., 2014). Naltrexone would need to be carefully titrated to avoid inducing aversive side effects

which deters use (Bouza et al., 2004). A further limitation of this combination approach is the risk of diversion of buprenorphine and its abuse liability.

Nevertheless, these data highlight the potential of combination buprenorphine/naltrexone as an antidepressant treatment strategy and provides an alternative route to achieving a shorter-acting safe and effective κ -antagonist.

ACKNOWLEDGEMENTS

This research is funded by the Government of Saudi Arabia through a PhD scholarship (AA), the Royal Society (SJB) and NIDA DA07315 (SMH).

Figure Legends

Figure 1. Antinociceptive effects of buprenorphine (Bup, 1mg/kg) and U50,488 (U50, 10 mg/kg) are blocked by naltrexone (NTX) in the mouse tail withdrawal assay. The time course of the experiments is shown (A,C, E). Bar charts highlight the antagonist effects of naltrexone (NTX) at 60 min post-administration of agonist (B, D). (A,B) Naltrexone (NTX) dose-dependently blocked buprenorphine-induced antinociception (** $p < 0.01$; *** $p < 0.001$ Bup compared to saline control; \$\$ $p < 0.01$; \$\$\$ $p < 0.001$ Bup alone compared to combination Bup + NTX 1 and 3 mg/kg). (C,D) Naltrexone (NTX) dose-dependently blocked U50,488-induced antinociception (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ U50 compared to saline control; \$\$ $p < 0.01$; \$\$\$ $p < 0.001$ U50 alone compared to combination U50 + NTX 1 and 3 mg/kg). (E) Duration of κ -antagonist effects of naltrexone alone or naltrexone/buprenorphine combination. Significant blockade of U50,488 induced antinociception is evident at 1 h post-administration and reversed by 24 h. At 8h post-administration, the combination of buprenorphine/naltrexone, produced a significant potentiation of U50-488-induced antinociception (^^ $p < 0.001$ compared to all other treatment groups; ### $p < 0.001$ for all treatment groups compared to NTX/Bup/saline controls; && $p < 0.01$ compared to NTX/Bup/saline controls and compared to NTX/Saline/U50 group). (F) The irreversible μ -antagonist CCAM (3 mg/kg) administered 24 h before testing blocked the NTX/Bup mediated potentiation of U50,488-induced antinociception at 8h post-administration (* $p < 0.05$ compared to U50 alone). All values are mean \pm SEM, $n = 5$ per group.

Figure 2. Locomotor activity in the open field in mice treated with buprenorphine (Bup, 1mg/kg) alone and in combination with naltrexone (NTX) (0.3, 1 and 3 mg/kg). All values are mean \pm SEM, $n = 5$ per group.

Figure 3. Conditioned place preference to buprenorphine (Bup, 1 mg/kg) in mice, in the presence and absence of naltrexone (NTX) (1 and 3 mg/kg). In a 900 s test, animals in all treatment groups did not show preference for either chamber during habituation (pre-conditioning). After 6 days of conditioning, buprenorphine significantly

increased the time spent in the drug-paired chamber. Values are mean \pm SEM, $n = 8$ per group. $*p=0.05$ vs pre-condition group.

Figure 4. Effects of buprenorphine (1mg/kg) and naltrexone (1mg/kg), alone or in combination, in the mouse forced swim test. The SSRI fluoxetine (20 mg/kg) was administered as a positive control. (A) All compounds under test produced antidepressant-like effects in the forced swim test. (B) The irreversible μ -antagonist CCAM (3mg/kg), administered 24h before buprenorphine, did not block the antidepressant-like effects of buprenorphine in the forced swim test. Data are expressed as mean \pm SEM ($n=10$ per group) of time spent swimming, climbing and immobile during the last 4 min of a 6 min swim test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to saline.

Figure 5: Effects of buprenorphine (1mg/kg) and naltrexone (1mg/kg), alone or in combination, in the mouse novelty-induced hypophagia task. The latency to drink milk in both the home and novel cage environments is shown. (A) The SSRI fluoxetine (20 mg/kg) was administered as a positive control and the selective κ -antagonist norBNI (10 mg/kg) shown for comparison ($n=10$ per group). (B) The irreversible μ -antagonist CCAM (3 mg/kg) blocked the effects of buprenorphine (1 mg/kg) on latency to drink in the home cage, but not the novel cage ($n=9$ per group). Data are mean \pm SEM. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared to saline. $\#p < 0.05$ and $###p < 0.001$ for novel cage comparison to home cage. $^^^p < 0.001$ compared to buprenorphine alone.

Figure 6: Effects of buprenorphine (1mg/kg) and naltrexone (1mg/kg), alone or in combination, in the mouse elevated plus maze (A,B,C) and light-dark box (D,E,F). The benzodiazepine diazepam (2 mg/kg) was included as a positive control. The time spent in the open arms (A), number of entries into the open arms (B) and total ambulation (C) in the elevated plus maze are shown ($n=10$ per group). The time spent in the light box (D), in the dark box (E) and total ambulation (F) in the light dark box

are shown (n=18 per group). All values are the mean \pm SEM. * p < 0.05 compared to saline.

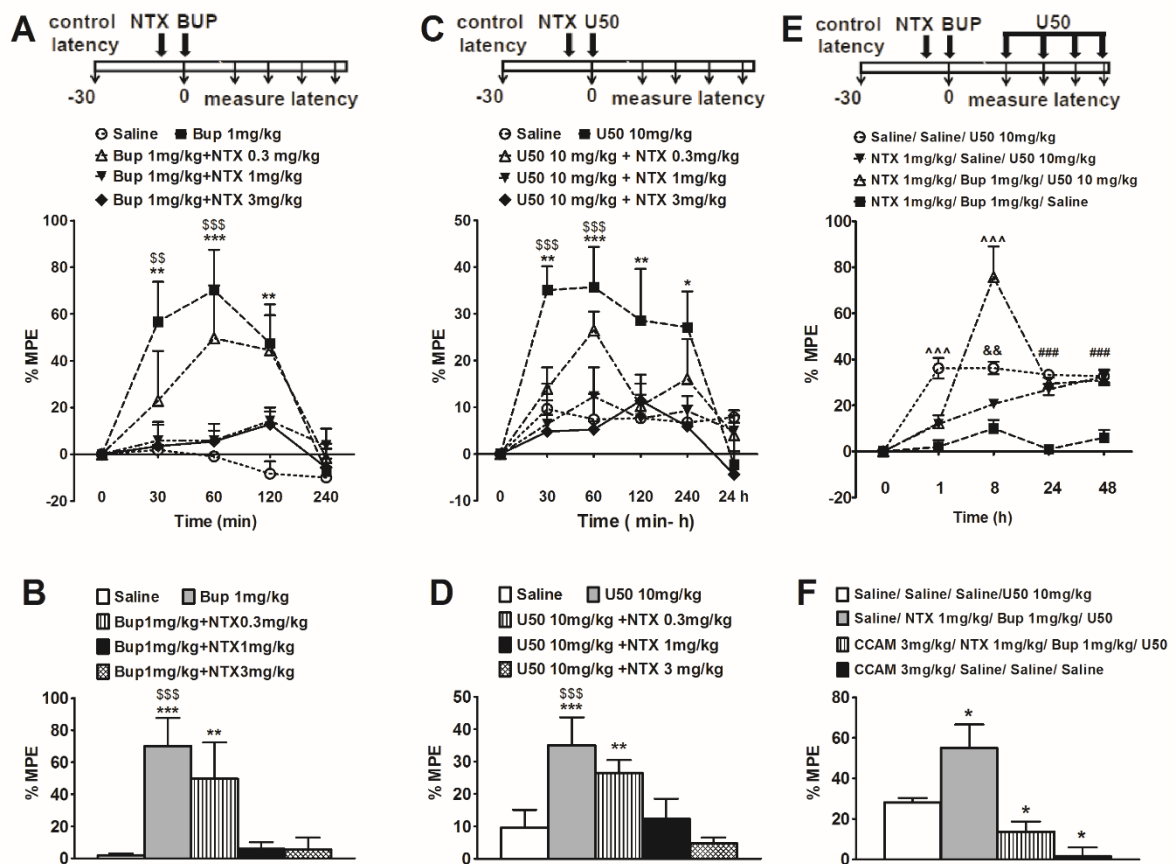


FIGURE 1

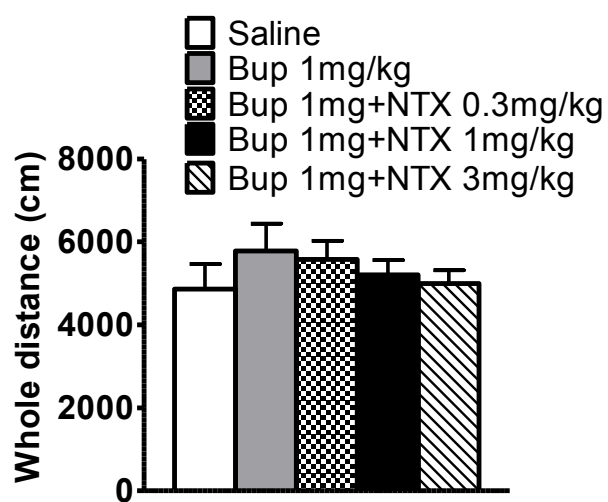


FIGURE 2

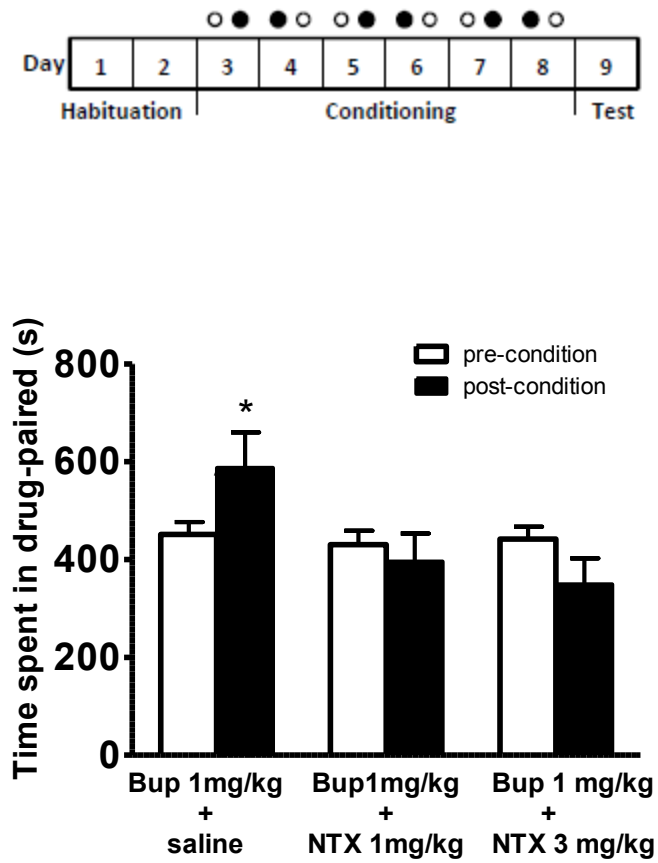


FIGURE 3

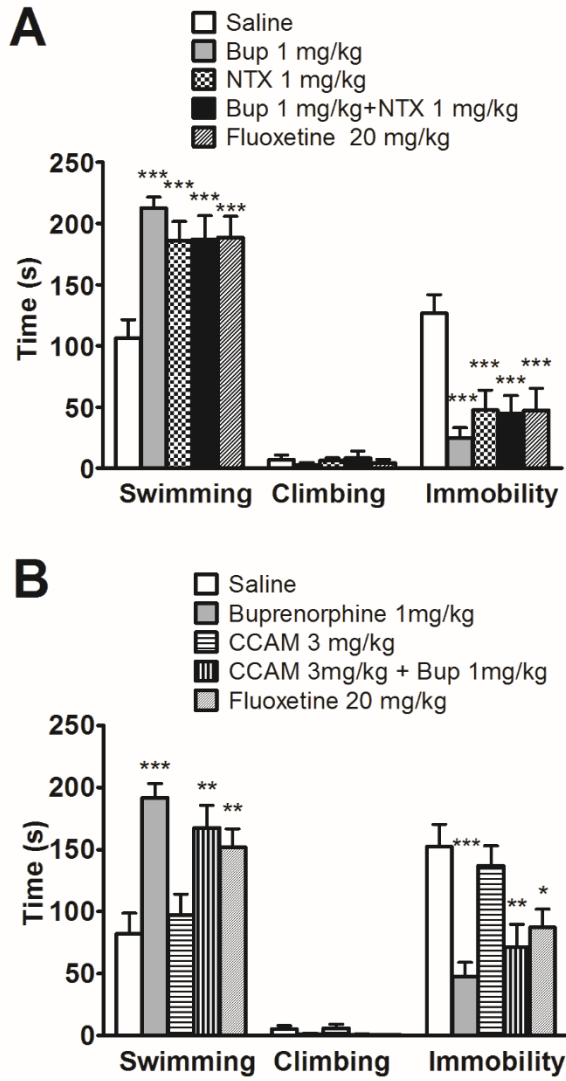


FIGURE 4

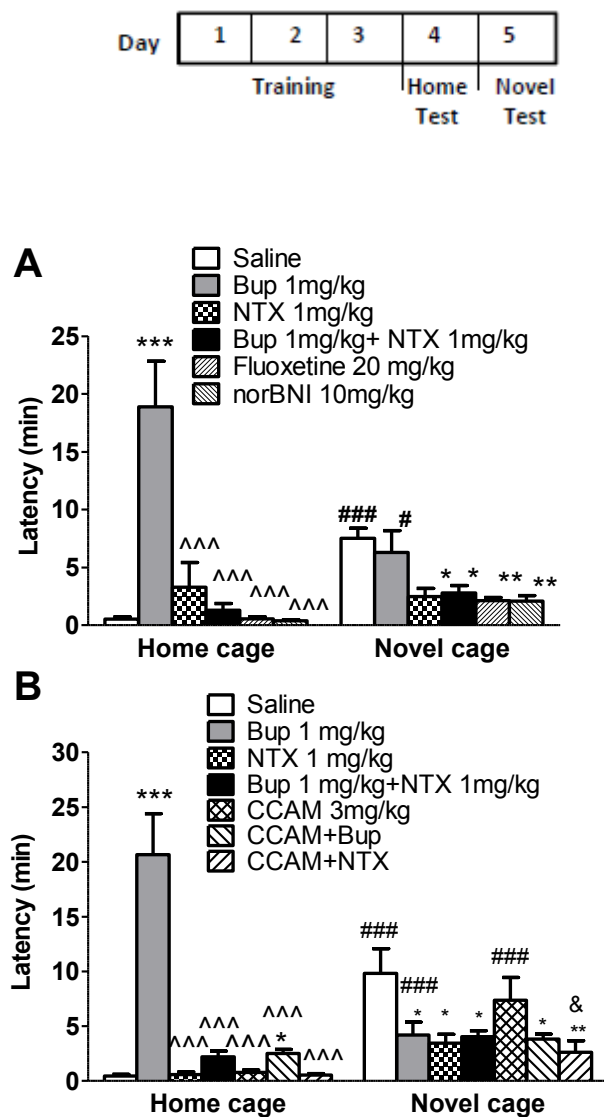


FIGURE 5

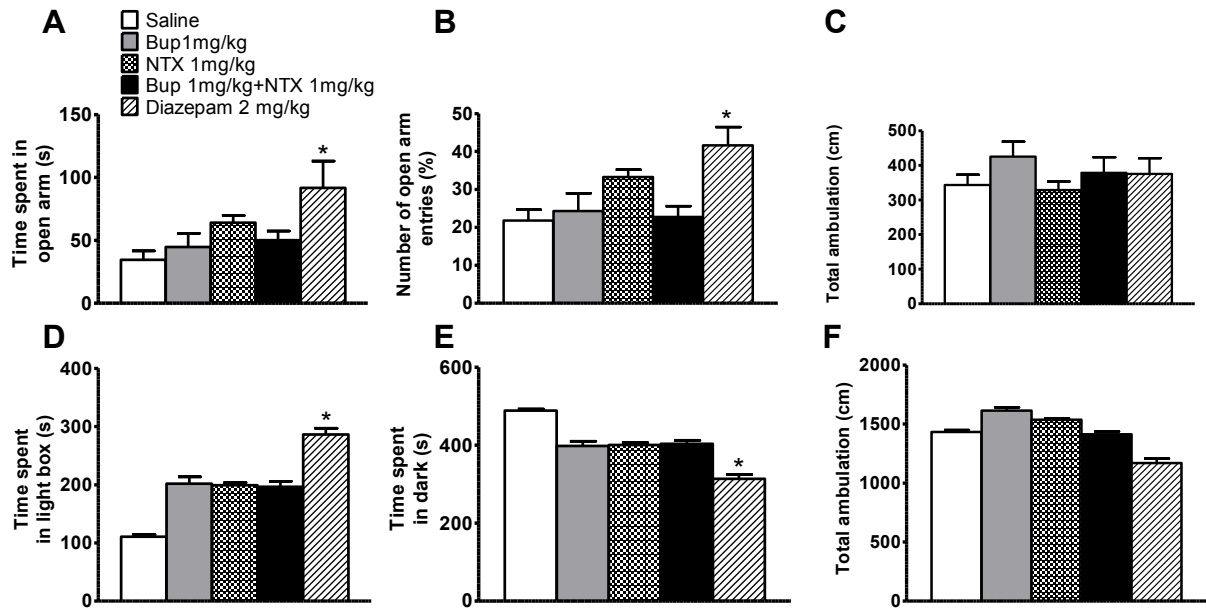


FIGURE 6

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