TITLE

Nalmefene is effective in reducing alcohol seeking, treating alcohol-cocaine interactions and reducing the alcohol-induced histone deacetylases gene expression in blood

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Author Contributions

JC-C, AG, and JA L-M made the conception and design of the study and drafted the manuscript. JC-C, VE-A, KMB and EG acquired, analyzed and interpreted the data. RN, FRdeF, RM and AG interpreted the data and made critical revisions for the manuscript.
SUMMARY

Background and purpose. The opioid antagonist nalmefene (selincro®) was approved for alcohol-related disorders by the European Medicines Agency in 2013. However, there have been no studies regarding the effectiveness of nalmefene in the combined use of alcohol and cocaine.

Experimental approach. Using operant alcohol self-administration in Wistar rats and qRT-PCR, we evaluated (a) the dose-response curve for subcutaneous and oral nalmefene; (b) the effects of nalmefene with increasing concentrations of alcohol; (c) the efficacy of nalmefene on cocaine-induced increases in alcohol responses; and (d) the gene expression profiles of histone deacetylases (Hdac1-11) in peripheral blood in vivo and in the prefrontal cortex, heart, liver and kidney post mortem.

Key results. Subcutaneous (0.01, 0.05, 0.1 mg/kg) and oral (10, 20, 40 mg/kg) nalmefene reduced dose-dependently the operant responses for alcohol up to 50.3%. Nalmefene systematically reduced alcohol self-administration independently from alcohol concentration (10%, 15%, 20%). While cocaine caused an increase of approximately 40% in alcohol responses, this result was fully reversed with a low dose of nalmefene (0.05 mg/kg). Additionally, alcohol caused a general increase in Hdac gene expression in blood, which was reduced in Hdac3, 8, 5, 7, 9, 6, 10 by nalmefene. In the other tissues, alcohol and nalmefene either did not alter the gene expression of Hdacs, as in the prefrontal cortex, or the gene expression showed a tissue-Hdac-specific effect.

Conclusions and Implications. Nalmefene might be effective in the treatment of the combined use of alcohol and cocaine, and Hdacs gene expression in peripheral blood might be used as a biomarker of alcohol use and drug response.

Abbreviations

Hdac, histone deacetylase.

qRT-PCR, Real-Time Quantitative Polymerase Chain Reaction
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CONFLICTS OF INTEREST

Nalmefene was provided by the pharmaceutical company Lundbeck (Copenhagen, Denmark) as a gift but no financial support from Lundbeck was received for these studies. AG has received consultancy fees and research grants from Lundbeck outside the submitted work.
INTRODUCTION

Nalmefene, formerly known as nalmetrene, is an opioid antagonist that structurally differs very little from the µ-opioid antagonist naltrexone. Nalmefene is obtained by the replacement of the ketone group at the 6-position of naltrexone with a methylene group (Hahn and Fishman, 1975). This subtle structural change seems to explain the increased affinity of nalmefene to the µ-opioid receptor and its longer half-life compared to naltrexone (Weinstein et al., 1971). Animal preclinical studies demonstrated that nalmefene was effective in reducing alcohol consumption and that it could be used as an alternative pharmacotherapy to naltrexone (June et al., 1998). In 2013, the results from several clinical trials demonstrated that nalmefene could be used to reduce alcohol consumption in patients with alcohol dependence (van den Brink et al., 2013; Gual et al., 2013; Mann et al., 2013). As a result, selincro® (nalmefene hydrochloride dehydrate), marketed by the Danish pharmaceutical company Lundbeck, was approved for alcohol-related disorders by the European Medicines Agency in 2013 (EMA).

In 2015, the first clinical experiment in which nalmefene was used for the treatment of cocaine-related behaviours (i.e., cocaine craving) was published (Grosshans et al., 2015). These authors found that nalmefene caused an abatement of craving for cocaine and could avoid relapsing into cocaine consumption. This is an interesting point since it has been estimated that for patients with an alcohol disorder the likelihood of having other addictive disorders were seven times greater than in the rest of the population (Regier et al., 1990). In fact, a diagnosis of alcohol dependence may be made in 50-90% of cocaine-dependent subjects (Lacoste et al., 2010), and there are some studies that demonstrate that the concurrent abuse of alcohol and cocaine would increase the incidence of neurological and cardiac emergencies (Faroog et al., 2009). Also, the co-consumption of alcohol and cocaine is common amongst recreational drug users. The concurrent use of alcohol and cocaine increases between 47-58% during the weekend compared with non-weekend days (Rodriguez-Alvarez et al., 2015). Therefore, one of the aims of the present study was to examine the effects of the opioid antagonist nalmefene on alcohol seeking and to identify its effects on alcohol-cocaine interactions.

Several authors have suggested that alcohol and cocaine are linked to differential expression profiling of chromatin modification enzymes (Botia et al., 2012; Kenedy and Harvey, 2015), and that histone deacetylases (Hdac1-11) gene expression from peripheral
blood might be used as a biomarker of the pathophysiology of psychiatric disorders (Hobara et al., 2010). That means that the diagnosis, prognosis and pharmacological responses of alcoholic individuals could potentially benefit from the gene expression profile of Hdac used as a biomarker. In this line, we have recently demonstrated that an increase in Hdac gene expression within the peripheral blood was associated with chronic alcohol consumption and that pharmacological treatments could prevent alcohol-induced changes in genes involved in epigenetic mechanisms, such as DNA methyltransferases (Echeverry-Alzate et al., 2014; Lopez-Moreno et al., 2015). For those reasons, the second aim of this study was to investigate whether a gene expression profile of Hdac could be linked to the behavioural results obtained.

**METHODS**

**5.1. Group sizes, Randomization and Blinding**

The exact group size for each experimental group has been provided for every group/condition within the Figure Legends. All the values correspond to independent values, not replicates, including values from the alcohol and benzoylecgonine analysis and qRT-PCR experiments. The minimum group size was n=7 for the nalmefene group in the later experiments (Figures 6-9). Every animal was randomly assigned to an experimental group and the nalmefene treatment order was fully counterbalanced. As far as the experimenters should treat the animals everyday (by injection or orally) they were aware of the pharmacological treatments of each group. Nevertheless, the responses of the animals for obtaining alcohol were automatically registered by the software and the experimenters had no access to the administration panel. In addition, the statistical analysis was performed by different researchers.

**5.2. Normalization and Statistical comparison**

No data from the behavioural experiments were normalized. For the qRT-PCR experiments, the 18S ribosomal RNA gene was used as an internal control for normalization according to the method described by Schmittgen and Livak (2008) in Nature Protocols and mentioned in the text (see below, Further Methods subheading).

For the statistical comparisons, only independent values have been used and data are represented as the mean ± SEM. All the statistical analysis performed have been fully
described (e.g., mentioning when independent or repeated measures were used) and all the results and most relevant parameters from the ANOVA tests are shown in Table 1. The threshold for statistical significance was defined previously ($p<0.05$) and maintained throughout all the manuscript, and when relevant, a Bonferroni correction was applied (i.e., in the gene expression study).

5.3. Validity of animal species, Model selection, Ethical statement, Animal details

We used male Wistar rats in these studies due to the high knowledge of the physiology, genetics, behaviour and cognitive functioning of these animals. Furthermore, we used the operant alcohol self-administration model, which is characterized for having reliability, a high face validity and predictive validity for humans (Koob et al., 2003). Fifty-six male Wistar rats (Harlan, Barcelona, Spain) were used. Forty had access to alcohol and sixteen only had access to saccharin in the operant self-administration procedures. Rats were purchased at seven weeks old and they weighted 375-475 g at the start of the pharmacological treatments. All research was conducted in strict adherence to the European Directive 2010/63/EU and Royal decree 53/2013 (BOE, 2013) on the protection of animals used for scientific purposes. The Ethics Committee of the Faculty of Psychology of the Complutense University of Madrid approved the study. The ARRIVE guidelines (Kilkenny et al., 2010; McGrath et al., 2010) were followed in designing the experiments.

5.4 Experimental procedures, Housing and husbandry and Interpretation (3Rs)

The experimental procedures are detailed below, in the experimental design section. Since their arrival, animals were housed in groups of 4 per cage (transparent polycarbonate, 1.815cm$^2$, -Eco-Pure Premium bedding from Datesand, Manchester, UK-) in a specific pathogen free and temperature- and humidity-controlled environment (21±1°C), on a 12 h reverse light/dark cycle (lights off at 08:00 h). Experimental sessions were performed during the dark phase. Food and water were available ad libitum except as specified below. The method of killing was by decapitation in order to obtain tissues from brain, heart, liver and kidney. All efforts were made to minimize animal suffering and to reduce the number of animals used. For instance, in order to reduce the number of Wistar rats, the same animals were used throughout the four studies described here (see Figure 1).
Further Methods

Experimental design

**Study 1. Dose-response curves for subcutaneous and oral nalmefene**

The aim of this study was to investigate the effects of nalmefene on alcohol self-administration. For this purpose we used an animal model with a high predictive validity for humans: operant alcohol self-administration in rats (Koob et al., 2003). It has been reported that subcutaneous nalmefene is between 3,200 and 6,400-fold more potent than oral nalmefene (June et al., 1998). Therefore, we performed two dose-response curves for nalmefene. In the first, nalmefene was administered subcutaneously (0.01, 0.05, 0.1 mg/kg) 20 min before the test, and in the second, it was administered orally (10, 20, 40 mg/kg) 90 min before. Every dose of nalmefene was administered over four consecutive days using a within-subjects Latin-square design with a two-day washout period between every dose. After it was observed that nalmefene significantly reduced the number of alcohol responses, we evaluated the motor activity and motor strength/coordination to rule out any impairment induced by nalmefene.

**Study 2. The effects of nalmefene on a dose-response curve for alcohol**

After ruling out any motor/coordination impairment, we examined the effectiveness of nalmefene on increasing doses of alcohol. This premise is supported by the fact that the alcohol content in alcoholic beverages is linked to the risk of becoming a heavy drinker, to the severity of alcohol dependence and to the adherence to treatments for alcoholism (Jensen et al., 2002; Baltieri et al., 2009). For this study, we chose the medium subcutaneous and oral dose of nalmefene (0.05 and 20 mg/kg, respectively), because they were the lowest effective doses for reducing alcohol responses and the grams of alcohol per kilo of body weight. The alcohol content (10, 15 and 20%) was increased every four consecutive days and was followed by a four-day washout period of nalmefene.

**Study 3. The effects of nalmefene on alcohol-cocaine interactions**

Based on the results of study 2, in which 0.5 mg/kg of subcutaneous nalmefene was proven to be effective in reducing alcohol responses throughout all alcohol concentrations tested, we decided to use this dose of nalmefene for the study of alcohol-cocaine interactions. For this, we used the methodology previously employed by our group (Echeverry-Alzate et al., 2012; 2014). It is noted that cocaine (20 mg/kg, i.p.) was injected six hours after the self-
administration of alcohol. This means that the animals return to the operant chamber 18 h after the cocaine injection. Using this protocol, cocaine-induced place-conditioned motor sensitisation and motor hyperactivity in the operant ethanol chambers would be avoided (Antoniou et al., 1998; Stromberg and Mackler, 2005). After a 10-day period of daily injections of cocaine in which an increase in alcohol responses was observed, the animals were treated over four consecutive days with nalmefene before alcohol self-administration. Blood samples were collected in vivo immediately after alcohol self-administration on the fourth day to determine blood alcohol levels as well as the levels of benzoylecgonine, one of the two primary metabolites of cocaine.

To gain a deeper insight into the interaction between nalmefene and cocaine, following a new four-day washout period of nalmefene, the animals were treated with subcutaneous nalmefene 20 min before they were injected with cocaine. The goal was to differentiate between the effects of nalmefene before alcohol self-administration and before the cocaine challenge.

**Study 4. Gene expression profiles of Hdacs**

After a final eight-day washout period for nalmefene, we studied the gene expression profiles of Hdacs in peripheral blood in vivo. The interest in studying Hdac gene expression lies in its novelty as a mechanism that underlies excessive alcohol consumption (Pandey et al., 2008; Warnault et al., 2013) and its potential role for being used as an alcohol biomarker (Lopez-Moreno et al., 2015). This study focused on this second aspect. For this, the same experimental conditions and results described in Figure 6 were replicated for four more days, during which time nalmefene was again administered before alcohol self-administration. Blood samples from the rat tail were collected in vivo immediately after the alcohol self-administration session. Then, as a complementary study, they were collected post mortem to determine the gene expression profiles of Hdacs in the prefrontal cortex, heart, liver and kidney. The prefrontal cortex was chosen among other brain regions because of its major role in goal-directed behaviours, its contribution to the development and maintenance of alcoholism (Lu and Richardson, 2014; Pfarr et al., 2015), and because nalmefene has a very high occupancy of µ-opioid receptors in this brain area (Ingman et al., 2005). The animals were sacrificed immediately after tail blood collection and the necessary tissues were collected.
Drugs and general procedures for pharmacological treatments

Nalmefene (17-cyclopropylmethyl-4, 5α-epoxy-6-methyleneleptorphinan-3,14-diol) was provided as a hydrochloride dehydrate salt by the pharmaceutical company Lundbeck (Copenhagen, Denmark) as a generous gift. The doses of nalmefene were calculated based on the salt and administered either by oral gavage (p.o.) at a volume of 3 ml/kg or subcutaneously at a volume of 1 ml/kg, (s.c.) in the scapular region. Nalmefene-control animals were treated with water (p.o.) or saline (s.c.). According to its peak plasma concentration, nalmefene was injected subcutaneously or administered orally, 20 and 90 min, respectively, before alcohol self-administration. Because the absorption of nalmefene is affected by the presence of food in the stomach, the animals were deprived of food 12 h before the oral treatment with nalmefene or water (the control group) (EMA). The doses of nalmefene were chosen according to previous studies using Wistar rats that investigated the effects of nalmefene on alcohol self-administration (June et al., 1998; Walker and Koob, 2008).

Cocaine hydrochloride (Sigma-Aldrich, S.L., Madrid, Spain) was dissolved in physiological saline and injected intraperitoneally (i.p.) at a volume of 1 ml/kg. Cocaine-control animals were injected with saline. The cocaine dose is expressed as the salt and was selected according to previous results (Echeverry-Alzate et al., 2012; 2014). Alcohol solutions were prepared every four days from 96% alcohol (Alcoholes Aroca, S.L., Madrid, Spain).

Operant self-administration and motor/coordination experiments

Apparatus and procedure

The operant alcohol sessions were conducted in eight modular chambers enclosed in sound-attenuating cubicles (Med Associates Inc., St. Albans, VT, USA). The exhaust fans were inactivated because the fans increased the rate of alcohol evaporation. The chambers were equipped with two retractable levers located 7 cm above a grid floor on either side of a drinking reservoir positioned in the centre of the front panel of the chamber and 4 cm above the grid floor. The levers were counterbalanced to respond as the active lever (delivering 0.1 ml) or as the inactive lever. Auditory or visual cues were not presented at any time. Training was conducted as follows: the rats were placed on a restricted water intake schedule for 12 hours ranging from two to four days to facilitate the training in lever pressing. For the rest of the experiments, the animals had access to food and water ad libitum except as specified for the oral treatment with nalmefene. During the first four days of training, animals received a
1% w/v saccharin solution (Sigma-Aldrich, S.L., Madrid, Spain) in the dipper. Thereafter, the following sequence was used on a fixed-ratio 1 schedule of reinforcement: 0.2% saccharin for three sessions, 0.2% saccharin and 0.2% alcohol for three sessions, 0.16% saccharin and 2% alcohol for three sessions, 0.12% saccharin and 4% alcohol for three sessions, 0.08% saccharin and 6% alcohol for three sessions, 0.04% saccharin and 8% alcohol for three sessions, 0.02% saccharin and 10% alcohol for three sessions and 10% alcohol for the rest of the sessions. The treatment with nalmefene started 19 days after 10% alcohol. Then, after the second study, 20% alcohol remained until the sacrifice of the animals. All the operant self-administration sessions lasted 30 min under a fixed-ratio 1 schedule seven days a week for the entire study. Sixteen animals that had access only to saccharin (0.005%) and did not receive any pharmacological treatment during the study were used as the control group for the genetic expression experiments (the calibrator group – i.e., the non-alcohol-treated group).

The locomotor activity of the rats was assessed during 30 min using six custom-made 40 x 35 x 35 cm rectangular boxes, and the boxes were equipped with eight photocells arranged in two lines (four and eight cm above the floor) that detected the locomotor activity as beam breaks. Strength and motor coordination was evaluated by the Rod Suspension test. The rat was held by the base of the tail and suspended over a horizontal rod (2 mm in diameter) until it grasped the rod with both forepaws. The body of the rat was then slowly lowered below the rod and the animal had to support its body weight. The rod was suspended at a height of 60 cm above a soft blanket. The time from release of the suspended rat until it let go of the rod was the latency in seconds (maximum time allowed was 60s). There were two trials the day before test (habituation), with an intertrial interval of 4 min, and one trial in the test day, after treatment with Nalmefene or vehicle (Goettl et al., 2001; Ingram et al., 1994; Thullier et al., 1996).

**Alcohol and benzoylecgonine analysis**

To determine blood alcohol concentrations and benzoylecgonine, 250 µl of blood was collected from the rat tail vein into a capillary tube (Microvette CB 300 K2E) that contained EDTA dipotassium salt. Every rat was placed on a towel on a table and held gently in place. The end of the tail was held, fixed between two fingers, onto the table. Using a scalpel a diagonal incision of 2mm long was made, at 15mm from the end of the tail. The collection of the blood took approximately 90s as maximum. The whole blood was centrifuged for 15 min at 1,500 x g using a refrigerated centrifuge, and the plasma was stored at -80ºC until use. The
alcohol concentration was measured using the EnzyChrom ethanol assay kit following the protocol recommended by the manufacturer (Bioassay Systems, Hayward, CA, USA). All measurements were performed in duplicate. Benzoylecgonine, a main metabolite of cocaine, was measured using the Cocaine Metabolite Direct Elisa Benzoylcygonine Assay Kit, following the manufacturer’s instructions (Bio-Quant, Heidelberg, Germany).

**Real time quantitative PCR experiments**
Real-time quantitative PCR was performed using a LightCycler 480-II machine (Roche, Barcelona, Spain) with SYBR Green Real-time qPCR master mix (Applied Biosystems, Warrington, UK) and specific primers at 300-nm concentrations (see Supplemental Table 1). The melting curves analysis showed only a single clear peak, and the size of the PCR products were confirmed by agarose gel electrophoresis. A 10-fold dilution series of the template was used to amplify each gene to validate the efficiency of each assay and to confirm that the amplification efficiencies of the target and reference genes were comparable (indicated by a near-zero slope value for both the target and reference genes). The 18S ribosomal RNA gene was used as an internal control for normalisation. The saccharin-vehicle group (the non-alcohol-treated group) was used as a calibrator, and the 2–ΔΔCT method was used to analyse the expression data (Schmittgen and Livak, 2008).

Blood samples from the rat tail were collected in vivo using capillary tubes (Microvette CB 300 K2E) immediately after the alcohol self-administration session as described above. Total RNA was isolated from whole blood using Trizol LS Reagent (Life Technologies, Carlsbad, USA). Then, the animals were sacrificed by decapitation. Prefrontal cortex, heart, liver and kidney were collected and immediately dissected on ice and were quickly frozen on dry ice at -80 °C. Total RNA was isolated using Tripure Isolation Reagent (Roche) and was stored at -80 °C. One microgram of total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche).

**Statistical Analysis**
Four types of ANOVA were used according to the nature of the variables. Data from Figures 2, 3, 4A / 4C, 6A / 6B, and 7 were analysed using a two-way mixed ANOVA (within-subjects: either days or intervals; between-groups: treatment). Data from Figures 4B / 4D and 6D / 6E were analysed using a one-way ANOVA. Data from Figure 5 were analysed using a
three-way mixed ANOVA (within-subjects: days and ethanol concentrations; between-groups: treatment). The data from Figure 8 and Supplemental Figure 1 (SF1) were analysed using a two-way ANOVA for each tissue (between-groups: Hdac x treatment). Bonferroni correction was applied for each tissue (P=0.05/11=0.004), and thus, only P values ≤0.004 were considered significant. These analyses were performed after controlling for assumptions (e.g., Levene’s test to assess variance homogeneity among groups), and the anomalous values detected through the SPSS box plot analysis were discarded. Except for Figure 8 and Supplemental Figure 1, after confirming the significance of the primary findings using ANOVA, a significance level of $p<0.05$ was applied to all remaining statistical analyses. The SPSS statistical software package (version 20.0) for Windows (Chicago, IL, USA) was used for all statistical analyses. All ANOVA results are detailed in Table 1.

RESULTS

Study 1. Dose-response curves for subcutaneous and oral nalmefene

Subcutaneous and oral nalmefene reduced dose-dependent levels of alcohol responses (Figure 2A and Figure 3A) and the consumption of grams of alcohol per kilo of body weight (Figure 2B and Figure 3B). With respect to accumulated alcohol responses over a four-day period, the highest doses of subcutaneous and oral nalmefene led to reductions of 49.5% and 50.3%, respectively, in alcohol responses. The lowest doses of subcutaneous and oral nalmefene caused a significant reduction in alcohol responses, but they did not reduce the consumption of grams of alcohol per kilo of body weight. This finding suggests that the values of alcohol responses may be more sensitive to the effects of nalmefene under our experimental conditions.

As day-by-day alcohol responses, there were only some slight differences. While the highest dose of subcutaneous nalmefene revealed rather a flat slope (Figure 2A), the highest dose of oral nalmefene had a greater effect on the first day of treatment (Figure 3A). This effect was regarded as a significant interaction (see Table 1) and is consistent with clinical trials with alcohol dependent patients (Sinclair et al., 2014).

Throughout the washout periods for nalmefene, there were no significant differences between nalmefene doses, neither in the accumulated alcohol responses (Figures 2C and 3C) nor in the grams of alcohol per kilo of body weight (Figures 2D and 3D). Only an interaction between nalmefene and days was observed (see Table 1).
These results caused us to question whether the observed reduction of alcohol responses induced by nalmefene could be associated with a motor impairment. Therefore, we conducted an additional experiment to examine the spontaneous locomotor activity and the strength/motor coordination of the animals after the subcutaneous and oral nalmefene treatments (Figure 4). We did not find any significant effects of nalmefene on animal motor activity or coordination, though the subcutaneous dose of 0.05 mg/kg of nalmefene exhibited a tendency ($p=0.08$) to increase the animals’ strength/coordination (Figure 4B). See Table 1 for ANOVA results. Compared to the control group, at the end of the treatments, the oral doses of nalmefene of 20 and 40 mg/kg caused a significant reduction in the rat body weight of 1.87% and 1.75% respectively.

**Study 2. The effects of nalmefene on a dose-response curve for alcohol**

Figures 5A and 5B depict two essential effects. First, nalmefene continued to be effective in reducing alcohol consumption independent of the concentration of alcohol (10, 15 and 20% v/v). Second, as long as the concentration of alcohol continued to increase, the number of alcohol responses decreased (Figure 5A). However, it is important to note that Figure 5B shows that the animals maintained the grams of alcohol per kilo of body weight, thus suggesting a set-point for the psychoactive effects of alcohol.

Overall, it is concluded that both subcutaneous and oral doses of nalmefene were equally effective in reducing alcohol responses and grams of alcohol per kilo of body weight. The only exception was in the combination of oral nalmefene and 15% alcohol concentration (Figure 5B, upper-middle panel), where the dose of oral nalmefene caused a greater reduction in grams of alcohol per kilo of body weight than did the subcutaneous dose.

After the treatment was interrupted (washout period for nalmefene), they returned to levels of alcohol consumption similar to those of the control group. (For detailed ANOVA results, see Table 1. Note that a reduced version of the three-way mixed ANOVA is provided due to space limitations). At the end of this experiment, it was observed that the oral dose of nalmefene of 20 mg/kg reduced 2.5% the weight of the animals compared to the control group.

**Study 3. The effects of nalmefene on alcohol-cocaine interactions**

As presented in Figure 6A, nalmefene was effective for preventing an increase in alcohol responses induced by cocaine. While cocaine led to an increase of 40.5% in alcohol responses, nalmefene caused a reduction of 68.9% in the nalmefene group and 71.1% in the
cocaine+nalmefene group. However, and unexpectedly, when the treatment with nalmefene was interrupted, the cocaine+nalmefene group did not return immediately to the same levels of alcohol responses as the cocaine group (Figure 6B, washout period).

As anticipated, a strong positive correlation ($r_{(38)}=0.79, p<0.001$) was found between the blood alcohol levels and the number of alcohol responses (Figure 6C). Mean blood alcohol levels were between 38 and 43 mg% in the control groups and between 7.5 and 7.6 mg% in the nalmefene groups (Figure 6D). Nalmefene did not alter the metabolism of chronic treatment with cocaine at a dose of 20 mg/kg, as no change in benzoylecgonine levels was detected (Figure 6E).

To gain deeper insight into the interaction between nalmefene and cocaine, two additional experiments were performed. First, we administered nalmefene prior to the cocaine treatment (i.e., 18 h prior to the alcohol session), which is in contrast to the previous tests wherein nalmefene was given prior to alcohol self-administration. Under these conditions, nalmefene was not effective in reducing alcohol responses or in preventing an increase in such responses induced by cocaine (Figure 7A). Furthermore, nalmefene had no effect on cocaine-induced locomotor sensitisation (Figure 7B). Cocaine significantly increased the locomotor activity of the animals, between 150% and 166%. Thus, the 18% reduction induced by nalmefene was not significant. For detailed ANOVA results, see Table 1.

**Study 4. Gene expression profiles of Hdac1-11**

The gene expression profile of *Hdac1*-11 from peripheral blood *in vivo* is presented in Figure 8. Alcohol self-administration caused a general increase in the expression of all *Hdac* genes, though *Hdac* 1, 3 and 11 did not support the Bonferroni’s correction for multiple comparisons. The significant increase in *Hdac* gene expression was between 62% and 142%. However, nalmefene prevented such increases, except with respect to *Hdac* 2 and 4.

The results regarding the other tissues analysed post mortem were more heterogeneous (*Supplemental Figure 1*). Specifically, the gene expression of histone deacetylases was not affected by any treatment in the prefrontal cortex; alcohol caused a reduction in the expression of *Hdac* 9 in the heart which was prevented by nalmefene and nalmefene caused a reduction in *Hdac* 2 in the liver. Despite that nalmefene showed an increase of *Hdac* 1, 4 and 9, in the kidney, only *Hdac* 7 survived the Bonferroni’s correction ($p\leq0.004$).
DISCUSSION

Using a reliable animal model of alcohol self-administration, the four studies performed herein have replicated previous findings and provided new answers regarding the effectiveness of nalmefene in reducing alcohol use. The first study confirmed that subcutaneous and oral nalmefene reduced dose-dependent responses to alcohol and result in no significant motor/coordination impairment. In the second study, we demonstrated that nalmefene decreased alcohol consumption independent of alcohol content. Thereafter, we found that nalmefene reversed cocaine-induced increases in alcohol consumption. In the final study, we determined that nalmefene reduced Hdac3, 8, 5, 7, 9, 6, 10 gene expression in peripheral blood in vivo caused by alcohol self-administration. Accordingly, the discussion is divided into four brief sections corresponding to the studies above.

The results of the first study are consistent with previous works using operant alcohol self-administration in Wistar rats or using alcohol-preferring rats (June et al., 1998; June et al., 2004; Walker and Koob, 2008; Nealey et al., 2011). Until now, there is no evidence that nalmefene activates other receptors than the opioid receptors, mainly mu and delta (Soyka, 2014). Also nalmefene acts as a partial agonist at kappa receptors which activation would lead to an elevation of serum prolactin and a reduction of dopamine in brain regions implicated in alcohol addiction (Bart et al., 2005). In this line, it has been demonstrated that the effects of nalmefene on alcohol-motivated behaviours is explained by the blockade of opioid receptors within the nucleus accumbens and ventral tegmental area (June et al., 2004, Nealey et al., 2011). Since nalmefene acts as a full agonist at the mu-opioid receptor, it has been suggested that genetic variants in this receptor might cause different responses to opioid treatments. However, this is still under debate because the results are heterogeneous (e.g., Arias et al., 2008; Bilbao et al., 2015). Also it should be noticed that there are some studies that show that nalmefene reduces the intake of natural rewards such as saccharin and a highly preferred food (June et al., 2004; Cottone et al., 2008). Interestingly, to optimise nalmefene bioavailability, the subcutaneous injection has been proposed. According to June and colleagues (1998), nalmefene was between 3.200 and 6.400-fold more potent than the oral administration. Here, both subcutaneous 0.01 mg/kg and oral 10 mg/kg doses of nalmefene were effective in reducing responses to alcohol, thus indicating that under our experimental conditions, subcutaneous nalmefene injection was 1.000-fold more potent than was oral administration. To the best of our knowledge, there have been no publications in which clinical trials have investigated the injection of nalmefene for alcohol addiction. Our results...
support the consideration of a pilot trial of injectable nalmefene for alcohol dependence, as occurred with naltrexone, which resulted eventually in the approval of Vivitrol® by the FDA (Lee et al., 2012; Sullivan et al., 2013).

Longitudinal studies have shown that the risk of becoming a heavy drinker or of developing an alcohol-use disorder is associated with the alcoholic beverage preference. While the highest risk is associated with low and high alcohol contents, that is, beers and spirits/liquors, the lowest risk was associated with moderate alcohol content, such as wines (Jensen et al., 2002; Grønbæk et al., 2004; Flensborg-Madsen et al., 2008; Siegel et al., 2011). In our second study, the animals had access to different concentrations of alcohol. The increase in the concentration of alcohol was accompanied by a decrease in the number of responses to alcohol. Control animals managed to maintain the total amount of grams of alcohol per kilo of body weight. This result reveals that the animals adjusted their responses to alcohol according to a specific psychoactive dose of alcohol. Nevertheless, subcutaneous and oral nalmefene reduced the responses to alcohol as well as the total amount of grams of alcohol per kilo of body weight independent of the alcohol content. Therefore, it is assumed that, to some extent, nalmefene reduces the psychoactive alcohol set-point. Alcohol content is also considered a significant factor as previous findings have shown that spirit drinkers and beer drinkers differ in their adherence to pharmacological treatments. For instance, beer preferring drinkers exhibited a higher degree of adherence to topiramate and naltrexone than did spirit preferring drinkers (Baltieri et al., 2009). Moreover, it is relevant to note that in our experiments, 96% alcohol diluted in water was used. Future preclinical studies using nalmefene should consider increasing the ecological validity of the alcohol drinking solutions, for example, using commercial beers, wines and spirits.

Previously, we reported that repeated cocaine injections induced an increase in the responses to 10% alcohol (Echeverry-Alzate 2012, 2014). Herein, we extended this finding by showing that cocaine also increased the responses to a 20% alcohol solution. However, more interesting was the fact that nalmefene fully reversed the cocaine-induced increase in the responses to alcohol. Surprisingly, while in studies one and two, the effects of nalmefene on alcohol responses disappeared within the first two days after nalmefene withdrawal, herein, the nalmefene’s effects did not disappear until the sixth day (data not shown). This might suggest that nalmefene would be more effective in reducing alcohol consumption in groups with higher levels of alcohol use. As expected, blood alcohol levels were directly
linked to the number of alcohol responses, and these values are similar to previous reports using Wistar rats and operant alcohol self-administration (Gilpin et al., 2009). Benzoylecgonine, one of the two primary metabolites in cocaine (Schindler and Golberg, 2012), did not show significant differences between the groups after 18 h of cocaine administration, indicating that nalmefene did not directly affect cocaine metabolism.

Accordingly, we questioned whether treating the animals with nalmefene before the cocaine injection instead of treating the animals prior to introducing them to the operant alcohol chambers could prevent the cocaine-induced increase in responses to alcohol. This experiment revealed that nalmefene was not effective in preventing the effects of cocaine on alcohol self-administration nor did the nalmefene affect cocaine-induced locomotor sensitisation. In addition, we observed that nalmefene was not effective in reducing responses to alcohol when nalmefene was administered 18 h prior to alcohol exposure. This finding is consistent with nalmefene’s posology, which indicates that one tablet of nalmefene should be taken one to two hours prior to the anticipated time of drinking (EMA). Finally, it is noted that the mechanism by which repeated cocaine administration increases the responses to alcohol remains unknown. Without cocaine interaction, it has been demonstrated that nalmefene (40 mg/day) suppresses the craving for alcohol and alcohol-induced stimulation (Drobes et al., 2004). Under our experimental conditions, among other explanations, we cannot discriminate whether cocaine increases the craving for alcohol, or whether it increases the threshold for the rewarding effects of alcohol leading the animal to increase its consumption, or whether this increase is caused by cocaine abstinence. Yet other tentative explanations might be that, on one hand, as far as cocaine produces anxiogenic actions, the animals would increase their alcohol self-administration because of its anxiolitic effects (Hendler et al., 2013; Ettenberg et al., 2015). And on the other hand, as far as cocaine has anorectic properties, the increase in alcohol responses would be driven for certain caloric needs (Barson et al., 2011; Soares et al., 2010). Therefore, further studies should investigate the psychobiological mechanism that accounts for this finding.

HDACs have been implicated in many diseases (cancer, cardiovascular, psychiatric diseases, for review see Abend et al., 2015; Sun et al., 2013) and many efforts are currently deploying to investigate their role as possible biomarkers. For instance, several PET radiotracers have been developed for visualizing HDAC activity in Alzheimer’s disease (Couto and Millis, 2015) although gene expression from peripheral blood is one of the most promising biomarkers (Mizuarai et al., 2010). Furthermore, pharmacological treatments are
able to alter the activity of HDACs. For instance, valproic acid, which is used to treat epilepsy and bipolar disorder, is an inhibitor of HDACs (Al Ameri et al., 2014). In the final study, we aimed to evaluate the gene expression profiles of *Hdac* in peripheral blood *in vivo*. This is not a new concept. For example, some authors have demonstrated that the expression of *Hdac* (1-11) in peripheral blood was associated with the pathophysiology of mood disorders (Hobara et al., 2010). Herein, we have developed this idea further by examining alcohol self-administration because recent studies have provided evidence that HDACs were involved in alcohol-related behaviours (Warnault et al., 2013; Pandey et al., 2015). In this study, it is noted that we did not investigate the functionality of HDACs or the relationship between mRNA and protein levels. Rather, we investigated whether mRNA levels of HDACs could be used as a biomarker of alcohol use and drug response. Recently, we have demonstrated that the gene expression profiles of *Hdac* in peripheral blood *in vivo* after alcohol intake or intoxication are similar in humans and animals. In both cases, alcohol increased the expression of most *Hdac* (López-Moreno et al., 2015). Nevertheless, it is important to highlight that while the first exposure to alcohol reduces *Hdac* gene expression throughout several tissues (Kirkpich et al., 2012; Botia et al., 2012; Pandey et al., 2015), repeated alcohol exposure causes this reduction to disappear or results in an increase of gene expression (Lopez-Moreno et al., 2015). Not only have we replicated portions of these results, but in addition, we have demonstrated that nalmefene reduced *Hdac*3, 8, 5, 7, 9, 6, 10 gene expression the alcohol-induced increase of *Hdac* in peripheral blood *in vivo*. Taking into consideration the gene expression profiles from the prefrontal cortex, heart, liver and kidney, it seems that, on the one hand, peripheral blood would not be the most reliable sample regarding the changes in *Hdac* gene expression that occur in those tissues after alcohol consumption. Actually, blood showed a general increase in *Hdac* gene expression, whereas the other tissues showed a more tissue-*Hdac*-specific effect. On the other hand, it appears that peripheral blood would be among one of the best tissue samples to use as a biomarker of alcohol use and drug response.

### 6c. CONCLUSION

In conclusion, we have added to the amount of evidence regarding the efficacy of nalmefene on alcohol-related behaviours, which also includes the co-administration of cocaine, and identified the role of *Hdac* gene expression in peripheral blood *in vivo* as a putative biomarker for alcohol use and nalmefene treatment.
6d. REFERENCES


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### Tables of Links

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These Tables of Links list key protein targets and ligands in this article that are hyperlinked* to corresponding entries in [http://www.guidetopharmacology.org](http://www.guidetopharmacology.org), the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson et al., 2014), and are permanently archived in *The Concise Guide to PHARMACOLOGY 2015/16* (Alexander et al., 2015).


Figure 1. Experimental timeline

The animals were introduced daily to the operant alcohol self-administration chambers without exception. The washout periods refer always to nalmefene. There was not a washout period for cocaine. The orange bar indicates when motor activity and strength/motor coordination tests were performed. * denotes blood collection in vivo for the analysis of blood alcohol levels and levels of benzoylecgonine; # denotes evaluation of motor cocaine-induced locomotor sensitisation; & denotes blood collection in vivo for the analysis of Hdac gene expression.
Figure 2. The effects of SUBCUTANEOUS nalmefene on responses to alcohol and grams of alcohol per kilo of body weight (30min session)

Nalmefene was administered for four consecutive days with a washout period of two days between doses (Latin square design). According to its peak plasma concentration, nalmefene was injected subcutaneously 20min before the operant alcohol self-administration session (control group, n=16; nalmefene groups, n=24). (A, C) Data represent the mean ± SEM of the accumulated or day-by-day alcohol responses to 10% v/v alcohol, and (B, D) represent their corresponding grams of alcohol per kilo of body weight. ** p < 0.01, *** p < 0.001 compared with the vehicle group; ## p < 0.01, ### p < 0.001 compared with the lowest dose of nalmefene. There were no significant differences throughout the withdrawal of nalmefene (washout period).
According to its peak plasma concentration, nalmefene was injected orally 90min before the operant alcohol self-administration session (control group, n=16; nalmefene groups, n=24). (A, C) Data represent the mean ± SEM of the accumulated or day-by-day alcohol responses to 10% v/v alcohol, and (B, D) represent their corresponding grams of alcohol per kilo of body weight. ** p < 0.01, *** p < 0.001 compared with the vehicle group; ### p < 0.001 compared with the lowest dose of nalmefene; & p < 0.05 compared with the intermediate dose of nalmefene. There were no significant differences throughout the washout period. See figure legend 1 for methodological details.
Figure 4. The effects of nalmefene on motor activity and strength/motor coordination

Nalmefene was injected subcutaneously or administered orally (20 and 90 min, respectively, according to its peak plasma concentration) before each test. Motor activity (A, C), mean ± SEM of the accumulated beam breaks for the 30 min following nalmefene administration (A, n=10 per group; C, n=11, 10, 10, 10 per group). Rod suspension (B, D), mean ± SEM of the latency to fall in seconds (B, n=10 per group; D, n=11, 11, 10, 10 per group). There were no significant differences in motor activity or strength/motor coordination.
Nalmefene was injected subcutaneously or administered orally as previously described. The concentration of alcohol was increased every four days up to a maximum of 20% and was followed by a washout period of four days. (A-D) Data represent the mean ± SEM (n=12, 14, 14 per group). Note that while the number of alcohol responses decreased according to the increase in alcohol content, the grams of alcohol per kilo of body weight remained stable. *** p < 0.001 compared with the vehicle group; # p < 0.05, compared with the subcutaneous dose of nalmefene. There were no significant differences in the washout period.
Figure 6. The effects of nalmefene on cocaine-alcohol interactions (I): before alcohol self-administration

Cocaine (20 mg/Kg, i.p.) was injected daily 18 h before the operant alcohol self-administration session, and nalmefene was administered subcutaneously 20min before the alcohol session. (A, B) Data represent the mean ± SEM of the accumulated or day-by-day alcohol responses to 20% v/v alcohol (n=11, 7, 11, 11 per group). * p < 0.05, *** p < 0.001 compared with the vehicle group; # p < 0.05, ## p < 0.01, ### p < 0.001 compared with the cocaine group. (C) Scatter plot of alcohol responses during the 30min alcohol session and blood alcohol levels determined immediately after the test session. Alcohol responses were significantly correlated with the blood alcohol levels (n=11, 7, 11, 11 per group). (D) Nalmefene reduced the blood alcohol levels according to the reduction in the number of alcohol responses independent of cocaine treatment. *** p < 0.001 compared with the vehicle group; ### p < 0.001 compared with the cocaine group (n=11, 7, 11, 11 per group per group). (E) Benzoylecgonine (ng/mL) was examined 18.5 hours after cocaine administration and immediately after alcohol self-administration (n=10-10 per group). Nalmefene did not alter the metabolism of cocaine.
Figure 7. The effects of nalmefene on cocaine-alcohol interactions (and II): before cocaine administration

Nalmefene was injected subcutaneously 20min before the administration of cocaine (20 mgKg, i.p.). Nalmefene administered 18 h before alcohol self-administration was not effective in reducing alcohol responses or in preventing cocaine-induced increases in alcohol responses. Nalmefene was not effective in preventing cocaine-induced locomotor sensitisation. (A) Data represent the mean ± SEM of the accumulated or day-by-day operant alcohol responses to 20% v/v alcohol (n=11, 7, 11, 11 per group). * p < 0.05 compared with the vehicle group; # p < 0.05 compared with the nalmefene group. (B) Motor activity, mean ± SEM of the accumulated beam breaks for the 30min after cocaine administration (n=11, 7, 11, 11 per group). *** p < 0.001 compared with the vehicle group; #### p < 0.001 compared with the nalmefene group.
Figure 8. The effects of alcohol and nalmefene on *Hdac* gene expression in peripheral blood *in vivo*

The animals were treated with alcohol and nalmefene as described in Figure 6. Blood samples from the rat tail *in vivo* were collected immediately after alcohol self-administration. Data represent the mean ± SEM (n=9, 8, 7 per group) of the relative fold change obtained using the 2ΔCt method. * p≤0.004 compared with the control-saccharin group.
### Table 1. Results of the ANOVAs

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**Figure 6**

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**Figure 7**

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**Blood**

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