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Effects of oral loperamide on efficacy of naltrexone, baclofen and AM-251 in blocking ethanol self-administration in rats

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ABSTRACT

Naltrexone is a μ-opioid receptor antagonist that has been extensively studied for its ability to block the rewarding effects of ethanol. Opioid receptors are widely distributed within the gastrointestinal tract (GIT). Typically, naltrexone is administered by parenteral routes in nonclinical studies. We initially tested if opioid receptors within the GIT would influence the ability of oral naltrexone to inhibit ethanol oral self-administration in rats using the co-administration of oral loperamide, a peripherally restricted opioid agonist. As expected, oral naltrexone only had modest effects on ethanol intake, and the response was not dose-dependent. However in rats, treatment with loperamide prior to the administration of naltrexone resulted in a suppression of ethanol intake which approached that observed with naltrexone given by the subcutaneous (SC) route. Importantly, administration of loperamide prior to administration of naltrexone did not alter blood concentrations of naltrexone. We then evaluated if oral loperamide would enhance effects of baclofen (a $GABA_B$ receptor agonist) and AM-251 (a CB-1 receptor antagonist) and found that pre-treatment with loperamide did potentiate the action of both drugs to reduce ethanol self-administration. Finally, the specific opioid receptor type involved was investigated using selective μ- and κ-receptor antagonists to determine if these would affect the ability of the AM-251 and loperamide combination to block ethanol drinking behavior. The effect of loperamide was blocked by ALKS 37, a peripherally restricted μ-receptor antagonist. These data suggest an important role for opioid receptors within the GIT in modulating central reward pathways and may provide new insights into strategies for treating reward disorders, including drug dependency.

1. Introduction

Drug dependency is a major health issue in society. The rewarding effects of drugs, including ethanol, are thought to be mediated by drug-induced increases in β-endorphin that lead to increased release of dopamine (DA) within the mesolimbic system. Specifically, activation of μ-opioid receptors by β-endorphin located on inhibitory GABA neurons that synapse with DA nerve cell bodies within the ventral tegmental area (VTA) leads to elevations of extracellular DA within the nucleus accumbens (NAc) (Devine et al., 1993a; Xi and Stein, 1998). Consequently, opioid antagonists, such as naltrexone and nalmefene, have been extensively studied in a variety of animal models as treatments for drug dependency-related disorders. Moreover, formulations of naltrexone are approved for treatment of alcohol and opioid dependence using both oral and parenteral routes of administration (Garbutt et al., 2005; Greenstein et al., 1981; O'Brien et al., 1978, 1996).

The majority of published nonclinical studies using naltrexone and nalmefene have utilized intraperitoneal (IP) or subcutaneous (SC) routes of administration to demonstrate effects of these drugs on both behavioral and neurochemical end-points associated with administration of ethanol. When given by the SC route both naltrexone and nalmefene are effective in attenuating ethanol oral self-administration in both mice (Grahame et al., 2000; Middaugh et al., 1999) and rats (McGregor and Gallate, 2004; Stromberg et al., 1998). Although not well documented in the literature, when using animal ethanol self-administration models, naltrexone and nalmefene show poor efficacy when given by the oral route. For example, nalmefene has been reported (June et al., 1998) to be at least 3000 times less potent when given orally than by SC injection using a rat ethanol drinking paradigm. Naltrexone was reported to reduce ethanol self-administration when given by the oral route (Parkes and Sinclair, 2000), but the magnitude of the observed effect was

Abbreviations: GIT, gastrointestinal tract; GABA, γ-aminobutyric acid; DA, dopamine; NAc, nucleus accumbens; VTA, ventral tegmental area; nor-BNI, nor-binaltorphimine dihydrochloride; CHO, Chinese hamster ovarian.

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low relative to other reports using SC drug administration with similar drinking models (Stromberg et al., 1998). The poor efficacy of oral naltrexone and nalmefene is commonly thought to be associated with low systemic exposure associated with extensive first-pass hepatic metabolism of these drugs. However, we have reported in a preliminary communication (Dean et al., 2007) that the circulating concentrations of naltrexone achieved after oral administration should have been adequate to inhibit ethanol self-administration in rats to a greater degree than commonly observed. This led us to hypothesize that a possible physiological mechanism(s) localized within the gastrointestinal tract and/or enteric nervous system might limit the CNS effects of orally administered naltrexone and nalmefene in rodents. If true, then oral administration of a peripherally restricted opioid agonist might enhance the oral activity of these commonly used opioid antagonists.

Therefore, the present experiments were conducted to investigate the role of the peripheral opioid system in modulating ethanol oral self-administration in rats. Our initial experiments confirmed differences between the efficacy of SC and oral naltrexone. We then tested the hypothesis that co-treatment with loperamide (a peripherally restricted opioid agonist) would increase the effectiveness oral naltrexone in attenuating ethanol self-administration in rats. Because endogenous cannabinoid and GABAergic systems have important roles in modulating rewarding effects of drugs within the mesolimbic system (Devine et al., 1993b; Perra et al., 2005; Xi and Stein, 1998), we further explored the ability of oral loperamide to enhance the action of baclofen (a $GABA_B$ agonist) and AM-251 (a CB-1 receptor antagonist) which are known to inhibit ethanol self-administration in rodents. Finally, the opioid receptor(s) mediating the effects of loperamide was explored using selective peripheral opioid antagonist in combination with loperamide.

2. Materials and methods

2.1. Subjects

Outbred male Wistar rats (starting weight of 200 ± 25 g; Charles River Laboratory, Raleigh, NC) were used in these studies. All rats were housed in polypropylene cages with free access to food and water (except during short periods of water-deprivation overnight) required for training and testing. For all ethanol self-administration studies rats were individually housed. Animals used for pharmacokinetic studies were housed in pairs. The vivarium was maintained on a 12 h light: dark cycle (0700:1900) with a room temperature of 22 ± 3 °C and a relative humidity level of 45 ± 10%.

2.2. Drugs

Naltrexone hydrochloride, loperamide hydrochloride, R(+)-baclofen and *nor*-binaltorphimine dihydrochloride (nor-BNI) were purchased from Sigma-Aldrich, St. Louis, MO. AM-251 was purchased from Tocris Bioscience, Ellisville, MO. ALKS 37 was synthesized for Alkermes, Inc. by Peakdale Molecular (Chapel-en-le-Frith, UK).

2.3. In vitro pharmacological characterization of ALKS 37

ALKS 37 was evaluated for binding to human opioid receptors and functional activity using receptor binding and [^{35}S]GTPγS binding assays. The K_i (binding affinity) values for μ-, δ-, and κ-receptors were determined with a previously described method using a competitive displacement assay (Neumeyer et al., 2003). Membrane protein from Chinese hamster ovarian (CHO) cells that stably expressed one of three types of human opioid receptors was incubated with 12 different concentrations of ALKS 37 in the presence of either 0.25 nM [^{3}H]DAMGO, 0.2 nM [^{3}H]naltrindole or 1 nM [^{3}H]U69,593 (μ-, δ-, and κ-receptors, respectively). Nonspecific binding was measured by inclusion of 10 μM naloxone. Radioactivity was counted and IC_{50} values and K_i values of unlabelled compound were calculated.

The [^{35}S]GTPγS assay measures functional properties of a compound by quantifying the level of G-protein activation following agonist binding in studies using stably transfected cells, and is considered to be a measure of the efficacy of a compound. ALKS 37 was evaluated using a [^{35}S]GTPγS assay to determine whether it functions as an agonist, antagonist, partial agonist or partial antagonist (Bidlack and Parkhill, 2004). To determine antagonist activity, CHO cells with stably transfected human μ- and κ-receptors were exposed to the μ agonist DAMGO, or the κ agonist U50,488, respectively, with increasing concentrations of ALKS 37. Nonspecific binding was measured by inclusion of 10 μM GTPγS. Agonist activity was also directly evaluated in this assay using varying concentrations of ALKS 37 alone.

2.4. Ethanol oral self-administration model

Rats were trained to orally self-administer ethanol using a modified operant procedure (Samson et al., 1989). Each operant chamber (Coulbourn Instruments, Whitehall, PA) consisted of a rat test box containing a single lever with a white cue light, a tone (2.9 KHz Sonalert®) and a liquid dipper with a 0.1 cc cup. The operant chamber was located in an isolation cubicle with a ventilation fan and internal background white noise.

Rats were hand shaped over a 1 to 3 day period to lever press (Fixed Ratio 1) for a 0.1% saccharine solution following overnight water deprivation. Once lever pressing behavior was established, water was again freely available in their home cage. A saccharine fading procedure was then utilized to initiate ethanol drinking. Rats were started on 5% ethanol in 0.1% saccharine and the ethanol concentration gradually increased to 10% and the saccharine concentration was then decreased to 0.04% over the next 20–40 sessions. Briefly, the start of the session was signaled by the activation of the house light. A cue light above the lever was turned on and the rat was required to press the lever two times (Fixed Ratio 2) to receive 3-second access to the ethanol cocktail from the liquid dipper. The presented reinforcer was signaled by a 0.5 s tone and a light located in the dipper receptacle. There was a 5 second inter-trial interval. Programming of the session and data recording was made using Graphic State 3 software (Coulbourne) running on a Windows XP compatible computer. Each daily session (5 days per week) lasted 30 min. Rats that consistently drank a minimum of 0.6 g/kg/h of ethanol (approximately 60 bar presses in 30 min with a 10% ethanol in 0.04% saccharine cocktail) over a 4-week period were used in these studies. Approximately 60% of the rats which began training were able to meet this criterion.

For the experiments described below, assessments of drug effects were made following the administration of a single dose of each drug or combination of drugs being evaluated. The same rats were used repeatedly throughout these studies to control for intra-subject variability. There was a minimum of a two-day drug-free washout period between study arms. For experiments using subcutaneously administrated naltrexone, AM-251 or baclofen, rats were placed in the chambers 30 min following administration of these drugs for data collection. When naltrexone was given orally, data collection was initiated 1 h following dosing. For all these experiments, ethanol self-administration refers to oral self-administration or ethanol drinking behavior as the actual volumes of ethanol consumed or blood alcohol concentrations were not measured.

2.5. Experiments

All studies reported herein were completed under protocols approved by Alkermes Institutional Animal Care and Use Committee and were conducted in accordance with the Institute of Laboratory Animal Resources' "Guide for the Care and Use of Laboratory Animals"

(Institute of Laboratory Animal Resources (U.S.). Committee on Care and Use of Laboratory Animals.)

2.5.1. Experiment 1: effects of naltrexone following subcutaneous and oral administration on ethanol self-administration

This study was conducted to characterize the effects of SC and oral naltrexone on ethanol self-administration within our experimental paradigm. For the SC arm of the study, doses of naltrexone (0, 0.05, 0.1, 0.5, 1.0, 3.0 and 6.0 mg/kg) were prepared daily in saline (1 mL/kg). For the oral arm of the study, doses of naltrexone (0, 2.5, 5 and 10 mg/kg) were dissolved in water (1 mL/kg). For each route, a minimum of five rats was used for each dose level. Oral doses were based on previous PK studies conducted by Alkermes and plasma concentrations would bracket those achieved with the 0.1 to 1 mg/kg SC doses.

2.5.2. Experiment 2: effects of loperamide on the ability of oral naltrexone to attenuate ethanol self-administration

The objective of this experiment was to determine if pretreatment with loperamide would alter the ability of oral naltrexone to attenuate ethanol self-administration. Rats were assigned to one of four treatment groups: a) vehicle only (n = 7); b) loperamide (n = 7); c) naltrexone (n = 7); and d) naltrexone and loperamide (n = 10). Loperamide (3 mg/kg) was prepared using 3% methylcellulose in water and given orally 30 min prior to administration of naltrexone. Naltrexone (10 mg/kg) was dissolved in water and administered orally 30 min prior to testing.

For the pharmacokinetic arm of this study, rats (n = 8 per treatment group) received a single PO (10 mg/kg) dose of naltrexone alone or in combination with loperamide (3 mg/kg) dose of loperamide. Blood samples (250 μL) were collected from the tail vein at −1, 0.5, 1, 1.5 and 2 h post naltrexone dosing. Plasma concentrations of naltrexone were determined by LC/MS-MS using a previously described method (Dean et al., 2008).

2.5.3. Experiment 3: effects of loperamide on the ability of baclofen and AM-251 to attenuate ethanol self-administration

This study was conducted to determine if loperamide would potentiate the activity of non-opioid drugs that have been previously shown to inhibit ethanol self-administration in rats. Baclofen and AM-251 were selected based on our previous experience with these drugs using our ethanol oral self-administration paradigm. The doses chosen for baclofen (1 mg/kg) and AM-251 (3 mg/kg) were intentionally selected to be sub-maximal, but previously shown to cause slight to moderate decreases in ethanol self-administration. Rats were assigned to receive: a) baclofen alone; b) baclofen plus loperamide; c) AM-251 alone; or d) AM-251 plus loperamide. Baclofen and AM-251 were administered subcutaneously (1 mL/kg) as suspensions in saline with 1% Tween 20. Loperamide (3 mg/kg in 3% methylcellulose) was given orally 30 min before treatment with baclofen or AM-251.

2.5.4. Experiment 4: evaluation of opioid receptors mediating effects of loperamide

The objective of this experiment was to determine which opioid receptors might be mediating the effect of loperamide. We selected the AM-251 and loperamide paradigm as a test system because the effect size of the combination of loperamide with AM-251 was large and robust. Two highly selective opioid antagonists were utilized: nor-BNI (for κ-receptors; (Portoghese et al., 1987)); and ALKS 37 (a peripherally restricted μ-receptor antagonist). Rats received: a) each of the drugs being used alone (AM-251, loperamide, nor-BNI or ALKS 37); b) the combination of AM-251 and loperamide; and c) the combination of AM-251 and loperamide with either nor-BNI or ALKS 37. Loperamide and AM-251 were prepared and administered as described for experiment 3. Nor-BNI (10 mg/kg, SC) was dissolved in saline and dosed 24 h prior to testing. ALKS 37 (3 mg/kg, PO) was dissolved in water and given 30 min before treatment with loperamide or 60 min before the beginning of the ethanol self-administration test period when given alone.

2.6. Statistical analyses

Data were analyzed using either GraphPad Prism 5 or SAS version 9.2. Details for the analysis of individual experiments are provided with the description of results.

3. Results

3.1. Route of administration affects the efficacy of naltrexone

The dose–response relationships between naltrexone given either SC or orally on ethanol oral self-administration are shown in Fig. 1 as percent change from baseline. As a point of reference, the mean (±sem) number of lever presses for the non-drug baseline was 136.4 (±9.9) in the 30 min session (range from 105 to 182 lever presses). Although blood ethanol concentrations were not determined in these studies, this was approximately 1.06 (±0.08) g/kg/30 min of ethanol consumed (range from 0.82 to 1.4 g/kg/30 min). A clear dose-related inhibition of ethanol consumption following SC administration of naltrexone was observed in rats (Fig. 1A). Control studies (data not shown) demonstrate that this naltrexone effect is specific for ethanol as total responses to 0.1% saccharine (in water) was not altered. It should be noted that vehicle injection frequently resulted in an increase in drinking activity above an animal's average baseline. This apparent stress response was prevented by 0.05 mg/kg of SC naltrexone ($p<0.05$ when compared to saline control). At 0.1 mg/kg SC naltrexone reduced ethanol self-administration when compared to saline ($p<0.01$), but also resulted in a marked reduction in ethanol drinking from baseline values. At 3 mg/kg SC naltrexone resulted in a near maximal inhibition of drinking, at approximately 80% of the baseline. The ED_{50} for naltrexone following SC administration was 0.47 mg/kg. In contrast to the SC route, oral administration of naltrexone produced only a modest (19–26%) reduction in alcohol intake at doses ranging from 2.5 to 10 mg/kg (Fig. 1B) that was not statistically significant ($p>0.05$; all doses compared to saline). This slight reduction in ethanol intake was not related to the dose of naltrexone administered.

3.2. Effects of loperamide on the ability of oral naltrexone to attenuate ethanol self-administration

Results from this experiment are shown in Fig. 2. As reported for experiment 1, oral naltrexone (10 mg/kg) again resulted in about a 25% reduction in ethanol intake when compared to the vehicle group that was not statistically significant ($p>0.05$). In addition, oral administration of loperamide alone did not affect ethanol self-administration ($p>0.05$). However, there was a significant reduction in ethanol self-administration following treatment with loperamide and naltrexone ($p<0.05$) when compared to each of the other groups, including a greater reduction in ethanol drinking behavior than observed with naltrexone alone (63 vs 26%; $p<0.05$).

A separate group of rats were used to determine if oral loperamide altered systemic exposure to naltrexone. Plasma concentrations of naltrexone were similar (treatment and treatment × time; $p>0.2$) between rats receiving only naltrexone and those given loperamide and naltrexone (Fig. 3).

3.3. Loperamide enhances the activity of other non-opioid drugs know to inhibit ethanol self-administration

To test the idea that loperamide would increase the effectiveness of non-opioid compounds influencing reward pathways, we

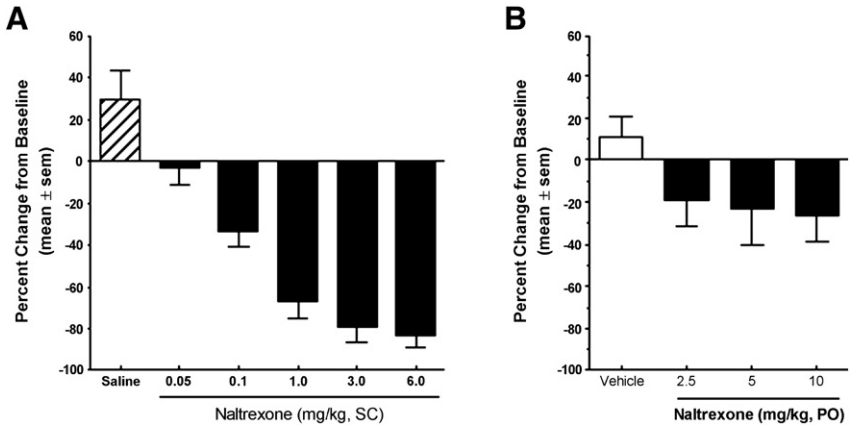

Fig. 1. Administration of subcutaneous (SC; Panel A) or oral (PO; Panel B) naltrexone (NTX) on oral self-administration of 10% ethanol (in 0.04% saccharine) in rats. Data were analyzed separately for each route of administration. A 1-way ANOVA was conducted using GraphPad Prism 5. If the model was significant, Dunnett's Multiple Comparison Test was utilized to compare each dose of naltrexone to the saline control group. SC and PO Following SC administration of naltrexone all doses (0.05 to 6.0 mg/kg) significantly reduced ethanol self-administration relative to saline ($p<0.05$) and the effect was dose-dependent reduction in drinking behavior. The ED_{50} of the SC dose–response curve was 0.47 mg/kg of naltrexone. Oral administration of naltrexone resulted in reduced ethanol drinking behavior at all doses tested; this reduction was not statistically different from the saline group.

investigated the effect of oral loperamide on the ability of SC baclofen (a $GABA_B$ agonist) or AM-251 (a cannabinoid receptor antagonist) to inhibit drinking activity in rats. Sub-maximal doses of baclofen (1 mg/kg) and AM-251 (3 mg/kg) were selected for combination with loperamide. Results are shown in (Fig. 4). The observed effect of baclofen alone was slightly greater than we had anticipated based on our own previous studies. However, the combination of loperamide with baclofen resulted in a greater reduction in ethanol intake than observed with baclofen alone ($p<0.05$). In contrast to baclofen, the dose of AM-251 selected was slightly less effective resulting in only about a 15% reduction in ethanol intake. However, when loperamide was given before AM-251 there was an approximately 80% reduction in ethanol self-administration compared to baseline; much greater than observed with AM-251 alone ($p<0.05$).

3.4. Evaluation of opioid receptors mediating effects of loperamide

We decided to take advantage of the potent synergistic action between oral loperamide and AM-251 to investigate the opioid receptors mediating the action of loperamide. In this experiment we also verified that administration of loperamide alone would not affect ethanol self-administration. To investigate the possible role of κ receptors rats were treated with the selective κ antagonist nor-BNI. Results are shown in Panels A–B of Fig. 5.

Treatment with loperamide alone did not influence ethanol self-administration (Fig. 5, Panel A). As in experiment 3, AM-251 alone resulted in a slight but not statistically significant attenuation of ethanol drinking behavior, while the combination of loperamide and AM-251 reduced ethanol drinking by approximately 80% (Fig. 5, Panel B). Neither nor-BNI alone (Fig. 5, Panel A), nor the combination of nor-BNI and AM-251 (data not shown) altered ethanol self-administration. Finally, nor-BNI did not prevent the robust attenuation of ethanol self-administration following administration of both loperamide and AM-251 (Fig. 5, Panel B).

Next we examined the role of μ opioid receptors using ALKS 37, a selective and peripherally restricted antagonist. ALKS 37 bound to μ, κ and δ opioid receptors with K_i values (±SEM) of 1.3 ± 0.13, 7.7 ± 0.90 and 280 ± 21 nM, respectively. In the functional [^{35}S]GTPγS assay, ALKS 37 demonstrated approximately a 150-fold selectivity (±SEM) for μ opioid receptors over κ opioid receptors with an IC_{50} value of 52 ± 20 nM and an I_{max} value of $96\pm1.2\%$ in inhibiting [^{35}S]GTPγS binding stimulated with 200 nM DAMGO. Despite the relatively good binding at the κ receptor, the IC_{50} value in the functional assay

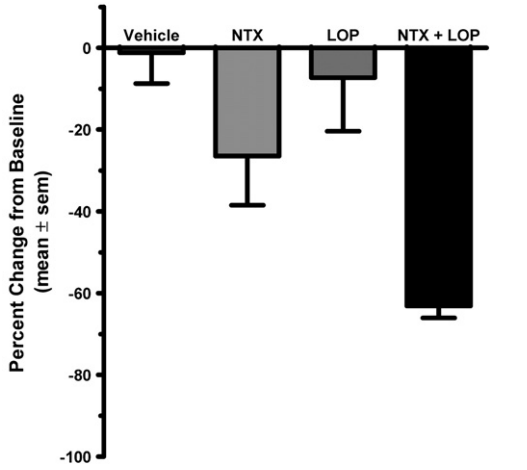

Fig. 2. Effect of oral naltrexone, with or without loperamide, on oral self-administration of 10% ethanol (in 0.04% saccharine) in rats. Data were analyzed using a 1-way ANOVA GraphPad Prism 5 followed by a Bonferroni's Multiple Comparison Test. Ethanol drinking behavior in rats treated with loperamide-only was similar to that observed for rats in the vehicle group. While oral treatment with naltrexone reduced ethanol self-administration relative to vehicle, this effect was not statistically significant ($p>0.05$). The combination of loperamide and naltrexone reduced ethanol self-administration significantly ($p<0.05$) when compared to each of the other three treatment groups.

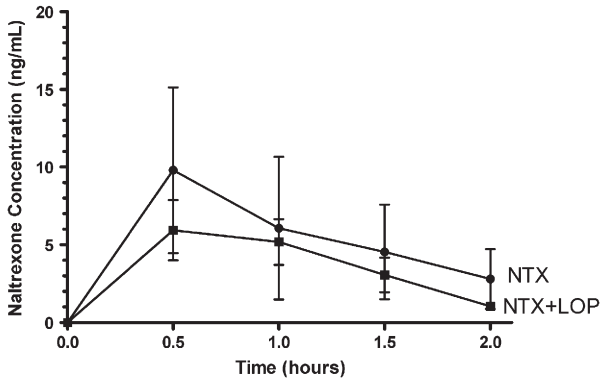

Fig. 3. Plasma concentrations of naltrexone following oral administration, with or without prior treatment with loperamide. Data were analyzed using the SAS Proc GLM procedure for repeated measures. Plasma concentrations increased following administration of naltrexone and were not affected by treatment with loperamide (time, $p<0.001$, treatment × time, $p>0.1$).

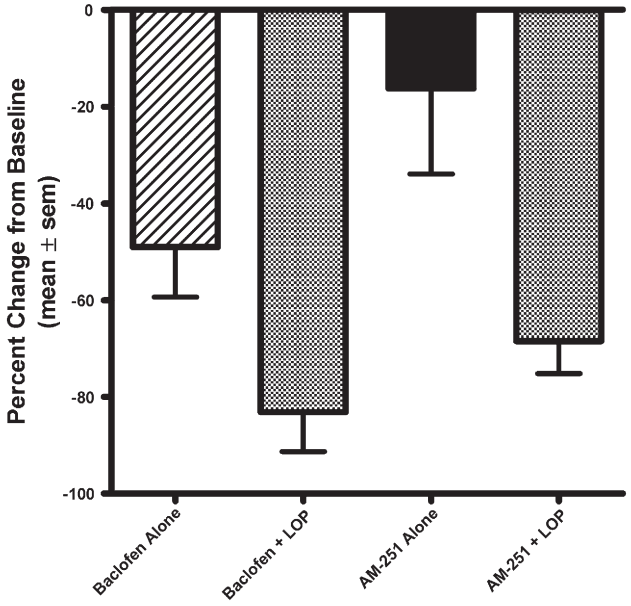

Fig. 4. Pre-treatment of rats with loperamide increases the ability of baclofen and AM-251 to inhibit ethanol drinking behavior in rats. The GraphPad Prism 5 unpaired *t*-test, with Welch's correction, was used to compare the effect of loperamide with baclofen and AM-251. Pre-treatment with loperamide (3 mg/kg, PO) resulted in a greater decrease in ethanol drinking behavior for baclofen ($p<0.05$) and AM-251 ($p<0.05$) when compared to these drugs alone.

was 7800 ± 530 nM with an I_{max} value of $88 \pm 1.4\%$ in [^{35}S]GTPγS binding stimulated with 100 nM U50,488.

Results on ethanol self-administration for the individual drugs and key combinations are shown in Fig. 6, Panels A and B. Administration of loperamide, ALKS 37 or AM-251 alone resulted in slight reductions in ethanol self-administration of 1, 14 and 13% from baseline, respectively (Fig. 6, Panels A and B). Once again pre-treatment with loperamide and then AM-251 resulted in approximately an 80% reduction in ethanol self-administration. This potent effect of combining loperamide and AM-251 was completely blocked by pre-treatment with ALKS 37 (Fig. 6, Panel B; $p<0.01$).

4. Discussion

The present studies contribute three important findings related to peripheral opioid mechanisms influencing ethanol oral self-administration (i.e., ethanol drinking behavior) in rodents. First, naltrexone is less effective when given by the oral route when compared to SC administration, and this decrease in effectiveness orally is not explainable simply on the basis of lower naltrexone bioavailability and reduced systemic exposure. Secondly, oral administration of loperamide enhances the ability of oral naltrexone, baclofen and AM-251 to inhibit ethanol self-administration. Finally, the action of loperamide is mediated by peripheral μ-receptors, likely localized in the GIT and enteric nervous system. Collectively, these findings support the idea of an opioid-mediated signaling pathway(s) originating within the GIT and enteric nervous system that modulates reward centers in the brain controlling ethanol self-administration.

Opioid antagonists have been extensively studied and shown to inhibit self-administration of rewarding drugs. Ethanol will increase the release of endorphins within the mesolimbic system, especially within the VTA and NAc shell. While naltrexone is frequently referred to as a non-selective opioid antagonist, recent evidence has challenged this concept showing that naltrexone is a μ-receptor antagonist, but also a partial agonist at kappa receptors (Bidlack and Parkhill, 2004). The μ- and κ-opioid receptors have opposing actions on mesolimbic dopaminergic neurons within the VTA and NAc, respectively (Spanagel et al., 1992). Consequently, the functional receptor activity profile of naltrexone would appear to be well balanced for reducing rewarding effects of drugs vis-à-vis inhibition of mesolimbic DA.

Although not well described in the published literature, it is widely recognized among investigators working in this field that oral treatment with opioid antagonists is not effective in preventing ethanol self-administration in rodents; consequently the majority of

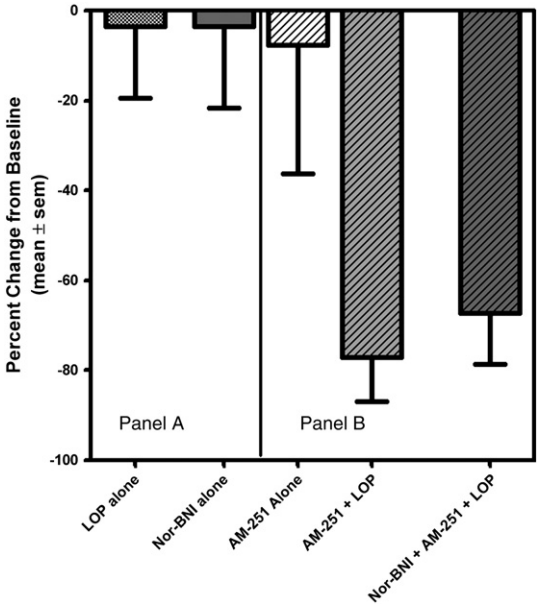

Fig. 5. Effects of loperamide, AM-251 and nor-BNI alone and in combination on ethanol self-administration. Neither loperamide nor nor-BNI resulted in any changes from baseline in ethanol drinking behavior. The GraphPad Prism 5 one-way ANOVA was used to compare the AM-251 alone, AM-251 and loperamide, and AM-251, loperamide and nor-BNI groups. When combined with AM-251, loperamide resulted in a significantly greater reduction in ethanol intake when compared to AM-251 alone ($p<0.05$); this effect was not influenced by nor-BNI ($p>0.1$).

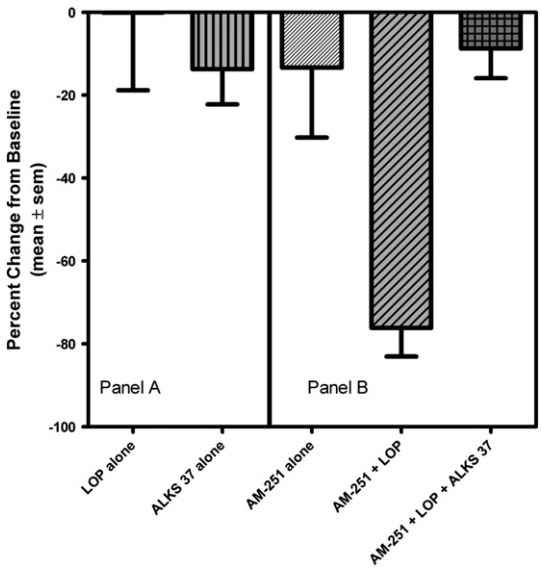

Fig. 6. Effects of loperamide, AM-251 and ALKS 37 alone and in combination on ethanol self-administration. Loperamide did not cause any changes from baseline in ethanol drinking behavior. Treatment with ALKS 37 resulted in a slight, but variable, reduction in ethanol intake of approximately 15% from baseline. The GraphPad Prism 5 one-way ANOVA was used to compare the AM-251 alone, AM-251 and loperamide, and AM-251, loperamide and ALKS 37 groups. When combined with AM-251, loperamide caused a significantly greater reduction in ethanol intake when compared to AM-251 alone ($p<0.01$) and this effect was blocked by pre-treatment with ALKS 37 ($p<0.01$).

the published studies using naloxone, naltrexone and nalmefene utilize parenteral routes of drug administration. The lower efficacy of orally administered opioid antagonists has long been held to be associated with extensive first pass metabolism of these drugs. For example, nalmefene was found to be at least 3000 times less potent when given orally than by SC injection in a rodent drinking paradigm (June et al., 1998). The poor oral activity of nalmefene was reported by these investigators to be due to first pass hepatic metabolism, but no pharmacokinetic data was provided supporting this conclusion. Others have reported that naltrexone was effective when given orally in suppressing ethanol drinking in rats (Parkes and Sinclair, 2000). However, the magnitude of the oral naltrexone effect on ethanol consumption was relatively poor when compared to other reports using SC administration with similar drinking paradigms. Also, recently it has been reported that naltrexone was less potent when given IP when compared to SC in blocking ethanol drinking in rats (Williams and Broadbridge, 2009). In our studies, the effect of SC naltrexone on ethanol oral self-administration was consistent with previous published reports using this route of administration. In addition, the slight inhibition of ethanol self-administration by oral naltrexone reported herein was similar to that achieved during the first one to three days of oral naltrexone treatment in an earlier study (Parkes and Sinclair, 2000). Thus, our data comparing the effect of route of naltrexone administration is consistent with both anecdotal reports and published literature.

It is clear that hepatic metabolism of naltrexone is extensive in rodents following oral administration, thereby limiting systemic exposure to the drug. A critical question for us was could hepatic metabolism account entirely for the poor efficacy of oral naltrexone in rats. Naltrexone bioavailability in rats is variable and has been reported to range from 5 to 20%. We have observed that based on PK modeling circulating concentrations of naltrexone following oral administration should result in greater suppression of ethanol self-administration than previously reported. These observations led us to question whether orally administered opioid antagonists were exerting a previously unrecognized pharmacological action that limited the effectiveness of naltrexone within the CNS. To evaluate this hypothesis we pre-treated rats with loperamide prior to administration of naltrexone.

Loperamide is a peripherally acting opioid agonist developed in the late 1970s by Janssen Pharmaceuticals and commonly used for the treatment of diarrhea. A key feature of loperamide is its low systemic exposure following oral administration. In rats loperamide has been shown to have good absorption into the tissues of the GIT, but less than 0.2% absorption into systemic circulation (Ooms et al., 1984). An interesting aspect of our initial studies was the apparent floor effect observed, where oral naltrexone could not reduce ethanol self-administration by more than approximately 25%. Given our previous findings based on PK modeling of experiments, published literature values for naltrexone, and the earlier findings for nalmefene (June et al., 1998) it appears that oral administration of opioid antagonists may modulate a GIT-brain pathway that is important for reducing the rewarding effects of ethanol. Treatment with loperamide enhanced the effectiveness of oral naltrexone without affecting circulating concentrations of the drug. A possible explanation for the enhanced activity of the combination of loperamide and naltrexone is that loperamide prevented local gastrointestinal effects of naltrexone, thereby allowing naltrexone reaching the CNS to exert a greater effect on reward centers leading to reduced ethanol consumption.

It was of importance to understand if activation of peripheral opioid receptors might enhance the activity of other drugs know to affect CNS reward centers. Therefore we evaluated if oral loperamide would potentiate the activity of non-opioid compounds that also have been shown to be effective in reducing ethanol self-administration in rodents. Baclofen, a $GABA_B$ receptor agonist, reduces alcohol consumption in both non-clinical models (Colombo et al., 2002; Maccioni et al., 2005) and clinical trials (Addolorato et al., 2002). As with naltrexone, the VTA is thought to be an important site of action for baclofen's inhibitory actions on ethanol intake. Pre-synaptic $GABA_B$ receptors are present on GABA neurons that provide tonic inhibition of dopaminergic neurons projecting to the NAc shell and activation of these receptors decreases DA release within the NAc. While the dose of baclofen used for our experiments provided a greater degree of inhibition of ethanol self-administration than we initially desired, it was clear that pretreatment with loperamide further enhanced the effectiveness of baclofen.

Endocannabiniods represent another class of endogenous ligands that modulate the rewarding effects of ethanol within the mesolimbic system (Perra et al., 2005). We utilized the CB-1 antagonist AM-251 to further evaluate the ability of loperamide to enhance the activity of drugs thought to work via inhibition of mesolimbic DA release. Like naltrexone and baclofen, AM-251 inhibits ethanol self-administration in rats (Femenia et al., 2010). In contrast to baclofen, the dose of AM-251 we used was less effective than anticipated. Nonetheless, there was a robust inhibition of ethanol drinking when rats were given loperamide prior to administration of AM-251. Importantly, we did not observe a reduction in ethanol self-administration when loperamide was given alone. At higher doses AM-251 can inhibit ethanol intake (Femenia et al., 2010), so the observed synergistic effect likely represents a leftward shift in the dose response curve for AM-251 in the ethanol self-administration model used for these experiments.

Our experiments with baclofen and AM-251 provided clear evidence that loperamide can potentiate the effects of non-opioid drugs on ethanol self-administration. While we think our data generated with oral loperamide and naltrexone strongly support the concept of a pharmacological mechanism for the interaction between these drugs, we cannot rule out a PK component due to the variability that is frequently observed in plasma concentrations of naltrexone following oral administration. However, results with baclofen and AM-251 reinforce the concept that a pharmacological mechanism is responsible for oral loperamide's ability to reduce ethanol self-administration for each of the three compounds studied. In addition, because loperamide did not impact ethanol self-administration by itself, it would appear that two or more converging pathways are require to achieve the reduction in ethanol intake observed with the drug combinations.

Generally loperamide is characterized as a μ-receptor agonist (Awouters et al., 1993). However, there are reports describing actions of loperamide mediated by κ-opioid receptors (Kojima et al., 2005; Kromer, 1995) and a few studies reporting effects via δ-opioid receptors (Dashwood et al., 1990; Giagnoni et al., 1983; Kojima et al., 2005). To investigate which receptor might be responsible for mediating effects of oral loperamide we focused on κ- and μ-opioid receptors, and used the robust effect of the loperamide and AM-251 on ethanol self-administration as the experimental paradigm. Nor-BNI is a potent selective κ-receptor antagonist (Portoghese et al., 1987) that has an extended duration of action. Administration of nor-BNI at doses that have been consistently shown to effectively block effects of κ agonists did not affect the ability of the loperamide and AM-251 combination to attenuate ethanol self-administration. Next we examined the role of μ-receptors using ALKS 37, which is functionally selective for this receptor, with very poor activity at κ-receptors. The effects of loperamide on AM-251-associated reduction in ethanol drinking were blocked by ALKS 37, and not by nor-BNI, suggests that activation of μ-receptors in the GIT is critical in mediating CNS-related modulation of self-administration behavior in rats.

P-glycoproteins (Pgp) are part of the ATP-binding cassette family of efflux transporters found primarily in the brain, GI system, gonads, kidneys and biliary system. Pgp transport certain hydrophobic

compounds out of the brain. Loperamide can penetrate the blood–brain barrier, although because it is a Pgp substrate, levels of loperamide within the CNS are kept low and central effects are limited (Ooms et al., 1984). While it is possible that some effects of loperamide observed in our studies could have been centrally mediated, even intravenous administration of loperamide (1 mg/kg) did not provide analgesia in rats (Emerich et al., 1998), suggesting significant CNS effects would be unlikely. A limitation of our studies is that we did not examine circulating concentrations of loperamide. When given orally, low systemic exposure can occur. Furthermore as with loperamide, systemic bioavailability of ALKS 37 is less than 0.1% following oral administration and studies using ^{14}C-labeled ALKS 37 demonstrated that when given orally greater than 95% of the drug remained associated with GIT tissues (Oleson et al., 2010). Therefore, when the low systemic absorption for both loperamide and ALKS 37 and the complete blockade of the loperamide effect by ALKS 37 are collectively considered, it would appear that the GIT is the primary site where loperamide is acting to enhance the activity of circulating naltrexone, baclofen and AM-251.

Beyond the involvement of μ-receptors, we have not ascertained the nature of the communication pathway(s) between the GIT and brain responsible for mediating effects of loperamide. Reduction of NAc DA release is a common feature of drugs that also inhibit ethanol self-administration, including naltrexone, baclofen and AM-251. In a preliminary communication we reported that oral naltrexone failed to alter ethanol and amphetamine induced-DA release within the NAc, but as observed in the ethanol self-administration model reported here, the combination of oral naltrexone and loperamide attenuated ethanol-induced increases in extracellular DA (Eyerman et al., 2010). Both neuroendocrine and neural pathways originating it the GIT could be affected by loperamide resulting in signaling to the CNS. For example, gut hormones such as CCK (Crespi, 1998; DiBattista et al., 2003; Kulkosky et al., 1993; Toth et al., 1990), and ghrelin (Addolorato et al., 2009; Jerlhag et al., 2009) have been reported to inhibit ethanol self-administration in rodent models. It is not known if loperamide affects the release of any of these gut hormones in rats.

Direct activation of neuronal pathways, such as afferent fibers extending from the viscera to the nucleus tractus solitarius (NTS) via the vagus nerve (Berthoud et al., 2001; Browning and Travagli, 2006; Ishii et al., 2010), could also be mediating the observed effects of loperamide. Indeed, neural circuitry connecting the NTS to the mesolimbic system has also been described (Delfs et al., 1998). Importantly, μ-receptors located within the intestinal wall have been reported to increase vagal afferent activity following administration of morphine; treatment with CTOP, a peripheral opioid antagonist, blocked morphine-induced increases in vagal afferent activity achieved with morphine (Banach et al., 2006). Finally, it has been reported that loperamide suppresses vagal-mediated release of pancreatic polypeptide in man (Riepl et al., 1996).

In conclusion, loperamide clearly enhances the ability of naltrexone, baclofen and AM-251 to inhibit ethanol self-administration in rats. This action of loperamide was shown to be mediated by μ-receptors, likely acting on receptors within the GIT and enteric nervous system. These data strongly support the concept of a neuroendocrine and/or direct neural pathway(s) linking the GIT to CNS centers that influence rewarding actions of ethanol.

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