Published in final edited form as:

Alcohol Clin Exp Res. 2019 June ; 43(6): 1077–1090. doi:10.1111/acer.14033.

Combination of clinically utilized kappa opioid receptor agonist nalfurafine with low-dose naltrexone reduces excessive alcohol drinking in male and female mice

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Abstract

Background: Nalfurafine is the first clinically approved kappa-opioid receptor (KOP-r) agonist as an anti-pruritus drug with few side effects in humans (e.g., sedation, depression and dysphoria). No study, however, has been done using nalfurafine on alcohol drinking in rodents or humans.

Methods: We investigated whether nalfurafine alone or in combination with mu-opioid receptor [MOP-r] antagonist naltrexone changed excessive alcohol drinking in male and female C57BL/6J (B6) mice subjected to a chronic intermittent access drinking paradigm (two-bottle choice, 24-h access every other day) for 3 weeks. Neuronal proopiomelanocortin enhancer (nPE) knockout mice with brain-specific deficiency of beta-endorphin (endogenous ligand of MOP-r) were used as a genetic control for the naltrexone effects.

Results: Single administration of nalfurafine decreased alcohol intake and preference in both male and female B6 mice in a dose-dependent manner. Pretreatment with nor-BNI (a selective KOP-r antagonist) blocked the nalfurafine effect on alcohol drinking, indicating a KOP-r mediated mechanism. Pharmacological effects of a five-dosing nalfurafine regimen were further evaluated: the repeated nalfurafine administrations decreased alcohol consumption without showing any blunted effects, suggesting nalfurafine did not develop a tolerance after the multi-dosing regimen tested. Nalfurafine did not produce any sedation (spontaneous locomotor activity), anhedonia-like (sucrose preference test), anxiety-like (elevated plus maze test), or dysphoria-like (conditioned place aversion test) behaviors, suggesting that nalfurafine had few side effects. Investigating synergistic effects between low-dose naltrexone and nalfurafine, we found that single combinations of nalfurafine and naltrexone, at doses lower than individual effective dose, profoundly decreased excessive alcohol intake in both sexes. The effect of nalfurafine on decreasing alcohol consumption was confirmed in nPE $-/-$ mice, suggesting independent mechanisms by which nalfurafine and naltrexone reduced alcohol drinking.

Conclusion: The clinically utilized KOP-r agonist nalfurafine in combination with low-dose naltrexone has potential in alcoholism treatment.

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Contributors: YZ designed the study, wrote the protocol, managed the literature searches and analyses, undertook the behavioral study, conducted statistical analysis, and wrote the manuscript. MJK contributed to the final versions of the manuscript. Both authors have approved the final manuscript.

Conflict of interest: All authors declare that they have no conflicts of interest.

Keywords

nalfurafine; KOP-r; excessive alcohol drinking; naltrexone; combined therapy; nPE knockout

INTRODUCTION

Excessive alcohol consumption affects the endogenous opioid systems. Specifically, activation of the kappa opioid receptor (KOP-r) system has been found to be involved in the negative reinforcing aspects of alcohol addiction [Herz 1997; Koob and Kreek 2007]. In rodents, KOP-r agonists reduce alcohol drinking and attenuate alcohol-induced reward [Sandi et al 1988; Lindholm et al 2007; Logrip et al 2009; Sperling et al 2010; Henderson-Redmond and Czachowski 2014] (also see [Anderson et al 2016; Rose et al 2016]). However, most “classic” KOP-r agonists like U50,488H and U69,593 induce strong sedation and dysphoria-like behavior, and such side effects limit their clinical use potential [e.g., Morani et al 2009]. Mesyl Salvinorin B (MSB), a novel selective KOP-r agonist with few side effects, has been recently found to attenuate drug intake and drug seeking behaviors [Simonson et al 2015; Zhou et al 2017, 2018a]. Together, these findings provide support for the important involvement of the KOP-r systems in the development of alcohol consumption and addiction.

Nalfurafine is a potent selective KOP-r agonist that has been well examined in rodents since 1990's [Nakao and Mochizuki 2009; Shigeki 2015]. In 2009, nalfurafine was the first clinically approved KOP-r agonist in Japan as an anti-pruritus drug for patients and it has been used for many years with few side effects in humans (e.g., sedation, depression and dysphoria) [Kumagai et al 2010, 2012; Pongcharoen and Fleischer 2016; Kamimura et al 2017]; especially in a recent post-market report on safety and efficacy of Remitch® (nalfurafine hydrochloride) in more than 3700 patients [Kozono et al 2018]. Pharmacologically, nalfurafine is an orally available small molecule and central-acting KOP-r agonist, with a long duration of action (biological half-life is 14–25 hours) [Rapaka and Sadée 2008]. Recently, nalfurafine has been characterized to have different signaling pathways than “classic” KOP-r agonists [Schattauer et al 2017; Liu et al 2018]. However, there is no study on the effect of nalfurafine on alcohol-associated behaviors in animal models. In the present study, therefore, we examined whether nalfurafine could reduce voluntary alcohol consumption in both male and female mice to explore its potential for a therapeutic compound for alcoholism.

Our novel hypothesis was that selective KOP-r agonist nalfurafine decreases excessive alcohol consumption with few side effects. For this purpose, we evaluated the pharmacological effect of nalfurafine using a chronic intermittent access (IA) drinking model, in which both male and female mice had access to voluntary alcohol drinking for 3 weeks in a two-bottle free-choice paradigm with alcohol intake every other day. Of importance, the mice exposed to this IA paradigm quickly developed high alcohol intake (15–25 g/kg/day), which constitutes an appropriate rodent model for studying excessive alcohol consumption [Hwa et al 2011; Zhou et al 2017, 2018a]. To determine the potential side effects, we further tested whether nalfurafine induced any anhedonia-like behavior in a

sucrose or saccharin drinking test, anxiety-like behavior in an elevated plus maze test, dysphoria-like behavior in a conditioned place aversion test or sedation in a spontaneous locomotor activity test.

Preclinical studies have consistently demonstrated that naltrexone, mu-opioid receptor [MOP-r] antagonist with a high affinity at MOP-r, and a mixed KOP-r partial agonist/antagonist with a low affinity, decreases alcohol's reinforcing and motivational properties and reduces alcohol intake, well consistent with clinical reports that naltrexone treatment in human alcoholics decreases alcohol drinking, craving and relapse [O'Malley et al 1992, 2002]. In recent preclinical investigations, several groups have studied the combinations of low-dose naltrexone with other compounds, including acamprosate, prazosin, MSB or V1b antagonist [Heyser et al 2003; Froehlich et al 2013; Zhou et al 2017, 2018b]. Of importance, the combination of low doses of naltrexone with these compounds show greater effects than either drug alone on reducing alcohol consumption in rodents, with less adverse effects. In the present study, our innovative investigation into the combination of nalfurafine with low-dose naltrexone could be particularly interesting, given that the two drugs have clearly different mechanisms of actions (KOP-r agonism for nalfurafine and MOP-r antagonism for naltrexone). Therefore, we specifically examined the combinations of nalfurafine and naltrexone with sub-effective doses of each drug. To maximize their effect on reducing alcohol drinking, these combinations with sub-effective doses may avoid potential tolerance development after repeated administration and minimize the potential side effects of each drug [Zhou et al 2017]. Our hypothesis was that by aiming at both KOP-r and MOP-r pathways implicated in alcohol addiction, the combination of the two clinically utilized compounds (nalfurafine+naltrexone) would enhance efficacy over the single-target approach [Zhou and Kreek 2018]. To further explore possible mechanisms for synergistic effects of the combination, we tested whether nalfurafine alone at a sub-effective dose would decrease alcohol intake in neuronal *Pomc* enhancers (nPE) knockout mice that has central beta-endorphin (relatively higher affinities at MOP-r and delta-opioid receptor than KOP-r) and MOP-r deficiency as a genetic control for the naltrexone effect [Lam et al 2015].

METHODS AND MATERIAL

Animals.

C57BL/6J (B6) mice.—Both male and female adult B6 mice purchased from The Jackson Laboratories were studied.

***Pomc* neuronal enhancers' knockout mice.**—Both male and female adult mice with targeted deletion of the two POMC neuronal enhancers (nPE^{-/-}) and the wildtype littermates (nPE^{+/+}) were studied.

Procedures.

1. Three-week Intermittent Access (IA) Drinking.—This model of alcohol consumption in B6 mice has been widely studied in many laboratories [e.g., Hwa et al 2011; Zhou et al 2017a]. In their home cages, mice had access to alcohol when food and water were always available. In this 2-bottle free-choice model, mice exposed to chronic alcohol

drinking every other day for 3 weeks. At 10:00 AM (3 hours after lights off) both the alcohol (15%) solution and water sipper tubes were put on the home cages. The left/right location of the tubes was randomly set to avoid any development of side preference. We recorded alcohol and water intake values at 3 time points (4, 8 and 24 hours after alcohol access) during the drinking days. Using these data, we calculated alcohol intake (i.e., g/kg) and relative preference for alcohol (i.e., alcohol intake/total fluid intake). In each sex, the mice were assigned randomly to the vehicle-treated and drug-treated groups, with similar alcohol intake 1 day before the test day.

1.1. Single administration of nalfurafine, naltrexone or their combination in the IA

model.: On the drinking test day, alcohol was available 30 min after an injection of nalfurafine (0.3–10ug/kg in saline, i.p.) or vehicle (saline), and water and alcohol intake values were recorded in male and female B6 mice. Or alcohol was available 10 min after an injection of naltrexone (0.3–3mg/kg in saline, i.p.) or vehicle (saline).

When the combined effects of nalfurafine and naltrexone were studied, B6 mice received the first injection of nalfurafine (0.3–3ug/kg) or saline followed by the second injection of naltrexone (0.3 or 1mg/kg) or saline 20 min later. Then alcohol was available 10 min after the second injection.

Then, we tested nor-BNI, a KOP-r antagonist, to pharmacologically block the KOP-r to confirm that the nalfurafine effects were mediated via KOP-r. Male B6 mice were pretreated with nor-BNI (2.5mg/kg) in saline (i.p.) 1 day before the drinking test, followed by one nalfurafine (10ug/kg) or vehicle injection 30 min before the drinking test.

Lastly, alcohol was available 30 min after an injection of nalfurafine (1ug/kg) or saline, and then alcohol and water intake values were recorded as described above in both nPE+/+ and nPE–/– mice of each sex.

1.2. Repeated administrations of nalfurafine in the IA model.: After 3-week IA, male B6 mice received 5 consecutive injections of nalfurafine (10ug/kg) or saline every other day before each drinking session (total 5 sessions). In each session, alcohol was available 30 min after one nalfurafine or vehicle, and then intake values were recorded.

2. Three-week drinking-in-the-dark (DID).—This model of alcohol consumption in B6 mice has been widely studied [e.g., Rhodes et al 2005; Zhou et al 2017a]. At 10:00 am (3 hours after lights off), the water bottle was switched to an alcohol (15%) bottle and kept for 4 hours before being switched back to the water bottle. We recorded alcohol intake values after 4 hours of alcohol access. On the drinking test day, alcohol was available 30 min after an injection of nalfurafine (3 or 10ug/kg) or vehicle.

3. Sucrose (caloric reinforcer) or saccharin (non-caloric reinforcer) drinking in B6 mice.—After 3-week IA, alcohol was replaced with sucrose or saccharin for 3 sessions to establish stable intakes. On the drinking test day, we recorded sucrose (4–16%) or saccharin (0.1–0.4%) and water intakes after 3 time points (4, 8 and 24 hours after single nalfurafine at 10ug/kg) in B6 mice of each sex.

We also tested the nalfurafine effect in alcohol-naïve B6 mice. The procedures were identical to the above experiment, except the mice were only exposed to sucrose or saccharin.

4. Single administration of nalfurafine on locomotor activity in B6 mice.—

Both male and female alcohol-naïve mice were injected with nalfurafine (10ug/kg, i.p.) or vehicle. Thirty min after the injection, mice were located into the appropriate chamber in a place conditioning apparatus for 30 min, and locomotor activity was assessed as the number of “crossings”. The behavioral experiment was run between 10:00 and 11:00 am.

5. Single administration of nalfurafine on anxiety-like behavior in B6 mice.—

Both male and female alcohol-naïve mice were injected with nalfurafine (10ug/kg, i.p.) or vehicle. Thirty mins after the injection, mice were placed on the central platform of the elevated plus maze facing an open arm and allowed to explore the maze for 5 min. The number of open arm entries and time spent in open arms were recorded manually as described (Maiya et al 2009). The behavioral experiment was run between 10:00 and 11:00 am.

6. Nalfurafine on “conditioned place aversion (CPA)” in B6 mice.—The behavioral experiment in male and female alcohol-naïve mice was run between 10:00 and 11:00 am using a place conditioning apparatus [Maiya et al 2009]. A dose of 10ug/kg (i.p.) nalfurafine was chosen because it was found to reduce alcohol drinking in mice in the above experiment. The protocol had 3 phases: (1) preconditioning: On day 1, the compartment doors were open, and mice could freely explore the entire apparatus. Their behavior was monitored for 30 minutes; (2) CPA training: During days 2 through 8 of CPA, mice were first injected with nalfurafine (10ug/kg, i.p.) and placed in a compartment for 30 minutes. On the alternative days, the mice were injected with saline and placed in the compartment opposite to where they received nalfurafine; and (3) CPA test: On day 9 (1 day after the last injection), the mice received no injections and were placed in the neutral, central gray compartment of the apparatus, and allowed to freely explore the entire apparatus. Their behavior was monitored for 30 minutes.

Data analysis.

Power analyses were performed to determine the number of mice required to provide statistically significant results, based on the levels of differences reported before [Zhou et al 2017a, 2018a]. In the experiments with single nalfurafine, naltrexone, their combinations or nor-BNI, the group differences in alcohol (or sucrose, saccharin) intake, water intake, total fluid and preference ratio in each sex were analyzed using 2-way ANOVA with repeated measures for treatment (vehicle vs drug) and for time interval (0–4, 5–8 vs. 9–24). For dose response analysis on nalfurafine alone and nalfurafine+naltrexone combinations, the group differences at the first 4-hour time point for alcohol intake and preference ratios were analyzed using 2-way ANOVA for treatments with different doses and for sex (male vs. female). In experiments with repeated administrations of nalfurafine, the group differences were analyzed using 1-way ANOVA. In nPE mice, the group differences in each sex were analyzed using 2-way ANOVA for genotype (nPE+/+ vs. nPE–/–) and treatments (vehicle

vs. drug). All the ANOVAs were followed by Newman-Keuls *post-hoc* tests. The accepted level of significance was $p < 0.05$ (*Statistica* version 5.5, StatSoft Inc., Tulsa, OK).

RESULTS

1. Single administration of nalfurafine alone decreased alcohol intake and preference in a dose-dependent manner after chronic IA in male and female B6 mice.

At a higher dose 10ug/kg (Figure 1), nalfurafine significantly decreased alcohol intake in males [2-way ANOVA, $F(1,13)=5.7$, $p < 0.05$] at 4 hours [*post-hoc* test $p < 0.05$] (Figure 1A, left) and in females [2-way ANOVA, $F(1,26)=8.2$, $p < 0.01$] at 4 hours [*post-hoc* test $p < 0.05$] (Figure 1A, right). There was a compensatory increase in water intake in males [$F(1,13)=6.1$, $p < 0.05$] at 4 hours [$p < 0.05$] (Figure 1B, left) and in females [$F(1,26)=5.4$, $p < 0.05$] at 4 hours [$p < 0.05$] (Figure 1B, right), resulting in unaltered total fluid intake in both males and females (Table 1A). At 10ug/kg dose, there was also a significantly reduction of preference ratio in males [$F(1,13)=6.8$, $p < 0.05$] at 4 hours [$p < 0.05$] (Figure 1C, left) and in females [$F(1,26)=6.2$, $p < 0.05$] at 4 hours [$p < 0.05$] (Figure 1C, right). After two low doses (1 and 3ug/kg) of nalfurafine, there was no effect on alcohol intake or alcohol preference ratio in either males or females (Figure 2).

Figure 2 presents the dose response of single nalfurafine administration (0, 0.3–10ug/kg) in alcohol intake and preference at the first 4-hour recording time. For alcohol intake (Figure 2A), there was a significant effect of nalfurafine [2-way ANOVA, $F(10,136)=17$, $p < 0.0001$], and *post hoc* analysis revealed that (1) compared with the control group, the nalfurafine-treated mice had less intake at 10ug/kg in both males and females [$p < 0.05$ for both]; and (2) the decrease at 10ug/kg was greater than that at 1ug/kg [$p < 0.05$ for both sexes]. For preference ratio (Figure 2B), there was a significant effect of nalfurafine [2-way ANOVA, $F(10,136)=14$, $p < 0.0001$], and *post hoc* analysis revealed that (1) compared with the control, the nalfurafine-treated mice had less preference at 10ug/kg in both sexes [$p < 0.05$ for both]; and (2) the decrease at 10ug/kg was greater than that at 1ug/kg [$p < 0.05$ for both sexes].

2. Single nalfurafine had no effect on sucrose or saccharin intake or preference in male or female B6 mice.

In these experiments, we verified the specificity of the nalfurafine effect on alcohol intake by testing the effects of 10ug/kg nalfurafine on sucrose (caloric reinforcer) or saccharin (non-caloric reinforcer) consumption in mice. As shown in Table 2, no significant effect of 10ug/kg nalfurafine on 4% sucrose (Table 2A) or 0.1% saccharin (Table 2C) intake or preference was found in either males or females at the first 4-hour recording time. There was no effect observed after 8 or 24 hours neither (data not shown). We also tested the effects of nalfurafine on other concentrations of sucrose (8% or 16%) or saccharin (0.2% or 0.4%) in both males and females ($n=4-5$) and did not observe any significant difference. Similarly, no effect of acute nalfurafine at 10ug/kg on sucrose or saccharin drinking was found in alcohol-naïve males and females (data not shown).

3. nor-BNI pretreatment blocked the effect of nalfurafine on reducing alcohol drinking in male B6 mice.

For intake (Figure 3), 2-way ANOVA showed significant effects of nalfurafine [$F(1,24)=7.7$, $p<0.05$], nor-BNI pretreatment [$F(1,24)=6.9$, $p<0.05$] and interaction between the nor-BNI pretreatment and nalfurafine [$F(1,24)=7.0$, $p<0.05$]. For preference ratio (Figure 3), 2-way ANOVA showed significant effects of nalfurafine [$F(1,24)=17$, $p<0.05$], nor-BNI pretreatment [$F(1,24)=11$, $p<0.05$] and interaction between the nor-BNI pretreatment and nalfurafine [$F(1,24)=7.9$, $p<0.05$]. At 10ug/kg, nalfurafine significantly decreased alcohol intake and preference ratio [$p<0.05$ for both], and pretreatment with nor-BNI at 2.5mg/kg blunted the nalfurafine effect.

4. Single administration of naltrexone decreased alcohol consumption in a dose-dependent manner after chronic IA in male and female B6 mice.

At two lower doses of naltrexone (0.3 or 1mg/kg), there was no effect on alcohol drinking in either sex. At a higher dose 3mg/kg, naltrexone significantly decreased alcohol intake in males [2-way ANOVA, $F(1,10)=7.7$, $p<0.05$] after 4 hours [$p<0.05$] (Figure S1A, left) and in females [2-way ANOVA, $F(1,14)=8.2$, $p<0.05$] after 4 hours [$p<0.05$] (Figure S1A, right). The naltrexone-treated mice showed a compensatory increase in water intake in males [$F(1,10)=6.1$, $p<0.05$] after 4 hours [$p<0.05$] and in females [$F(1,14)=5.9$, $p<0.05$] after 4 hours [$p<0.05$] (Figure S1B), resulting in unaltered total fluid intake (Table 1B). For preference ratio, two-way ANOVA showed a significant effect of naltrexone in males [$F(1,10)=9.3$, $p<0.01$] at 4 hours [$p<0.05$] (Figure S1C, left) and in females [$F(1,14)=10$, $p<0.01$] at 4 hours [$p<0.05$] (Figure S1C, right).

Like the previous reports [Zhou et al 2017, 2018a], naltrexone at this dose range (0.3–3mg/kg) had no effect on sucrose (4–16%) or saccharin (0.1–0.4%) consumption in either males or females (data not shown).

5. Single administration of low-dose nalfurafine combined with low-dose naltrexone decreased alcohol consumption in a dose-dependent manner after chronic IA in male and female B6 mice, with no effect on sucrose or saccharin drinking.

5.1. Nalfurafine combined with naltrexone on alcohol drinking.—As shown in Figure 2, single administration of nalfurafine (0, 0.3 or 1ug/kg) combined with naltrexone (0, 0.3 or 1mg/kg) decreased alcohol intake and preference in a dose-dependent manner in both sexes (data at the first 4-hour time point are analyzed together). There was no effect of nalfurafine at 0.3ug/kg dose combined with naltrexone at 0.3mg/kg in either sex. Combined with a higher dose of 1mg/kg naltrexone, however, nalfurafine at 0.3ug/kg significantly decreased both alcohol intake [$p<0.05$] (Figure 2A) and preference ratio [$p<0.05$] (Figure 2B) in both sexes, when compared with the vehicle control. Also, 1ug/kg nalfurafine with either 0.3 or 1mg/kg naltrexone significantly decreased alcohol intake [$p<0.05$ and $p<0.01$, respectively] (Figure 2A) and preference ratio [$p<0.05$ for both] (Figure 2B) in both sexes. The reductions at 1ug/kg nalfurafine with 0.3 or 1mg/kg naltrexone were greater than those at 1ug/kg nalfurafine alone for both sexes [$p<0.05$ and $p<0.01$, respectively].

Of note, combined with 0.1mg/kg naltrexone, other experiments also tested in both male and female mice using low doses of nalfurafine (0.3 or 1ug/kg), and no significance was found (data not shown).

Figure 4 presents alcohol and water intake values at 4, 8 and 24 hours following the combination dose (1ug/kg nalfurafine+1mg/kg naltrexone) in males and females. In males, the combination significantly decreased alcohol intake [$F(1,10)=5.2$, $p<0.05$] between 0–4 and 5–8 hour intervals [$p<0.05$ for both] (Figure 4A1) and preference ratio [$F(1,10)=5.5$, $p<0.05$] after 4 hours [$p<0.01$] (Figure 4C1). In females, the combination decreased alcohol intake [$F(1,14)=9.8$, $p<0.01$] between 0–4 and 5–8 hour intervals [$p<0.05$ for both] (Figure 4A2) and preference ratio [$F(1,14)=8.7$, $p<0.01$] between 0–4 and 5–8 hour intervals [$p<0.05$ for both] (Figure 4C2). The combination did not have any effect on total fluid intake (Table 1C).

At 3ug/kg nalfurafine with 0.3 or 1mg/kg naltrexone, alcohol and water intake in females are presented in Figure S2. Combined with 0.3mg/kg naltrexone, 3ug/kg nalfurafine significantly decreased alcohol intake [2-way ANOVA, $F(1,10)=5.6$, $p<0.05$] between 0–4 hour interval [$p<0.05$] (Figure S2A1). This combination significantly decreased preference ratio [$F(1,10)=5.5$, $p<0.05$] between 0–4 interval [$p<0.05$] (Figure S2C1). With 1mg/kg naltrexone, 3ug/kg nalfurafine decreased alcohol intake [2-way ANOVA, $F(1,14)=13.7$, $p<0.01$] between 0–4 and 5–8 hour intervals [$p<0.05$ for both] (Figure S2A2), coupled with a compensatory increase in water intake between 0–4 hour interval [$p<0.05$] (Figure S2B2). The combination also significantly decreased preference ratio [2-way ANOVA, $F(1,14)=11.2$, $p<0.01$] between 0–4 and 5–8 hour intervals [$p<0.05$ for both] (Figure S2C2). Neither combination dose had any effect on total fluid intake (data not shown).

5.2. No effect of single administration of nalfurafine with naltrexone on sucrose or saccharin drinking.—We tested the specific effect of 1ug/kg nalfurafine + 1mg/kg naltrexone combination on sucrose and saccharin drinking after chronic IA. After 4 hours, no effect of the combination (the most effective combination for reducing alcohol) on 4% sucrose or 0.1% saccharin drinking was observed in either sex (Table 2B, 2D). Similarly, there was no effect of the combination on other concentrations of sucrose (8% or 16%) or saccharin (0.2% or 0.4%) intake in either males or females ($n=4-5$). Finally, no effect of the combination on sucrose or saccharin in alcohol-naïve males or females was observed (data not shown).

6. Repeated administrations of nalfurafine did not developed tolerance on decreasing alcohol drinking.

To evaluate the effect of repeated nalfurafine on alcohol intake after 3-week IA, one group of males received 5 repeated administrations of 10ug/kg nalfurafine and the effects were compared among the 5 sessions (Figure 5). For intake, 1-way ANOVA showed a significant effect of nalfurafine (Figure 5A) [$F(5,24)=4.1$, $p<0.01$], and nalfurafine significantly decreased alcohol intake in all the sessions [$p<0.05$ for all], with no tolerance development. For preference ratio, 1-way ANOVA showed a significant effect of nalfurafine (Figure 5B)

[$F(5,24)=3.7$, $p<0.05$], and nalfurafine significantly decreased preference ratio in all the sessions [$p<0.05$ for all], without showing any tolerance.

7. Acute administration of nalfurafine at a sub-effective dose (1ug/kg) decreased alcohol intake after chronic IA in male and female nPE mice.

In nPE males after 4 hours, 2-way ANOVA showed significant effects of genotype [$F(1,20)=61$, $p<0.005$] and nalfurafine [$F(1,20)=5.6$, $p<0.05$] on alcohol intake (Figure 6A, left). *Post hoc* analysis showed that: (1) between genotypes, nPE $-/-$ had less intake than nPE $+/+$ [$p<0.05$]; and (2) nalfurafine decreased intake in nPE $-/-$ [$p<0.05$], but not nPE $+/+$. For water intake, 2-way ANOVA showed a significant effect of genotype [$F(1,20)=5.4$, $p<0.05$]. For alcohol preference, 2-way ANOVA showed a significant effect of genotype [$F(1,20)=37$, $p<0.01$], and nPE $-/-$ had less preference than nPE $+/+$ [$p<0.05$] (Figure 6B, left). Between 5–8 hours (Table S1A), 2-way ANOVA showed significant effects of genotype [$F(1,20)=33$, $p<0.01$], nalfurafine [$F(1,20)=5.0$, $p<0.05$] and interaction between genotype and nalfurafine [$F(2,20)=4.7$, $p<0.05$] on alcohol intake: between genotypes, nPE $-/-$ had less intake than nPE $+/+$ [$p<0.05$] and nalfurafine decreased intake in nPE $-/-$ [$p<0.05$]. There was no effect of nalfurafine between 9–24 hours (Table S1C).

In nPE females after 4 hours, 2-way ANOVA showed significant effects of genotype [$F(1,20)=49$, $p<0.005$] and nalfurafine [$F(1,20)=5.6$, $p<0.05$] on alcohol intake (Figure 6A, right). *Post hoc* analysis found that: (1) nPE $-/-$ had less intake than nPE $+/+$ [$p<0.05$] on alcohol intake; and (2) nalfurafine decreased intake in nPE $-/-$ [$p<0.05$]. For water intake, 2-way ANOVA showed a significant effect of genotype [$F(1,20)=5.5$, $p<0.05$]. For alcohol preference, 2-way ANOVA showed a significant effect of genotype [$F(1,20)=50$, $p<0.01$], and nPE $-/-$ had less preference than nPE $+/+$ [$p<0.05$] (Figure 6B, right). Between 5–8 hours (Table S1B), 2-way ANOVA showed significant effects of genotype [$F(1,20)=29$, $p<0.01$], nalfurafine [$F(1,20)=5.1$, $p<0.05$] and interaction between genotype and nalfurafine [$F(2,20)=4.6$, $p<0.05$] on alcohol intake: nPE $-/-$ had less intake than nPE $+/+$ [$p<0.05$], and nalfurafine further decreased intake in nPE $-/-$ [$p<0.05$]. There was no effect of nalfurafine between 9–24 hours (Table S1D).

8. Single administration of nalfurafine had no effect on alcohol intake after chronic 3-week DID in male or female B6 mice.

We also determined the effect of nalfurafine using the DID model (a short-access “binge” alcohol drinking model) and found no effect on alcohol intake in either male or female mice after nalfurafine at 3 or 10ug/kg (Figure 7).

9. Single administration of nalfurafine had no effect on locomotor activity in male or female alcohol-naïve B6 mice.

We further tested whether nalfurafine could induce sedation in mice at the dose required to attenuate alcohol drinking. The dose of 10ug/kg and injection schedule (30 min before the test) were based on the above alcohol drinking experiments. There was no effect of 10ug/kg nalfurafine on locomotor activity in either sex (Table 3).

10. Single administration of nalfurafine had no effect on anxiety-like activity in male or female alcohol-naïve B6 mice.

Male and female mice displayed relatively similar basal levels of anxiety-like behavior, as measured by both open arm entries and time spent in the open arms after vehicle injection in an elevated plus maze test (Table 4). In both male and female mice, the number of open arm entries and time spent in open arms were scored 30 min after nalfurafine at 10ug/kg, and there was no significant effect of nalfurafine.

11. Single administration of nalfurafine did not induce conditioned place aversion in male or female alcohol-naïve B6 mice.

There was no significant effect of nalfurafine at 10ug/kg on the time spent in either the nalfurafine-paired compartment or the saline-paired compartment (Table 5).

DISCUSSION

In the present study, we first determined the dose responses of nalfurafine, a clinically utilized KOP-r agonist, in decreasing alcohol consumption after chronic excessive alcohol drinking in an IA model or after chronic limited drinking in a DID model. In both male and female mice, nalfurafine at 10ug/kg dose (but not lower doses from 0.3 to 3ug/kg) decreased alcohol intake in the excessive drinking IA model (Figure 1), but not in the limited drinking DID model (Figure 7). Also, nalfurafine dose-dependently decreased alcohol preference after the excessive drinking IA model in both sexes (Figure 1). The decreasing effect of nalfurafine on excessive alcohol drinking was unlikely due to a general suppression of consumption or appetitive (anhedonic effect) behaviors, as we found that nalfurafine did not reduce sucrose or saccharin consumption (Table 2). In this study, we further confirmed that the effect of nalfurafine was KOP-r mediated, as the effect of nalfurafine on alcohol drinking was blocked by selective KOP-r antagonist nor-BNI (Figure 3). Consistent with previous studies in mice and rats, we confirmed sex differences in alcohol drinking, with higher alcohol intake in females in general [Becker and Koob 2016]. However, we observed a similar dose response of nalfurafine in both sexes (Figure 2). To our knowledge, this is the first description of nalfurafine, a clinically approved KOP-r agonist, on alcohol drinking, showing that nalfurafine reduced excessive alcohol consumption in mice with no sex difference. The new finding is in line with our recent results showing the reducing effect of KOP-r agonist MSB on alcohol consumption in mice [Zhou et al 2017].

Nalfurafine at 10ug/kg decreased alcohol drinking (Figure 1) but did not produce any anhedonia-like behavior in the sucrose preference test (Table 2A), anxiety-like behavior in the elevated plus maze test (Table 4), dysphoria-like behavior in the conditioned place aversion test (Table 5) or sedation in the spontaneous locomotor activity test (Table 3). Our result is well consistent with many early studies in rodents with similar dose ranges of nalfurafine, but different from many “classic” KOP-r agonists with several side effects. Specifically, low doses of nalfurafine (<40µg/kg) do not produce conditioned place aversion, sedation, anxiety-like or depression-like behaviors [Nagase et al 1998; Suzuki et al 2004; Inan et al 2009; Liu et al 2018], though high doses (>80µg/kg) were reported to induce aversion [Lazenka et al 2018]. In humans, unlike other KOP-r agonists, nalfurafine does not

produce strong hallucinogenic or dysphoric effects [Kumagai et al 2010, 2012; Pongcharoen and Fleischer 2016; Kamimura et al 2017; Kozono et al 2018]. With few adverse effects, the most common side effect of low-dose nalfurafine seen in clinical trials was insomnia in 10–15% of patients [Shigeki 2015]. Recent preclinical studies support the notion that nalfurafine exhibits different signaling properties than “classic” KOP-r agonists with anhedonia-like, dysphoria-like or drug-seeking effects (like U50,488H and U69,593) [Schattauer et al 2017; Liu et al 2018]. As such, more research is needed to clarify the distinct mechanisms of this compound. Our study is also in line with growing research into novel KOP-r ligands with fewer side effects [White et al 2015; Brust et al 2016].

Many studies have found that U50,488H or U69,593 increases alcohol consumption, triggers alcohol-seeking behavior or induces “relapse” drinking (see update reviews [Anderson and Becker 2017; Zhou and Kreek 2018]). Our present finding that nalfurafine (a KOP-r full agonist) decreased, rather than increased, drinking, seems opposite to the studies using the above “classic” KOP-r agonists [Figure 1]. After excessive alcohol drinking, however, the brain KOP-r/dynorphin systems are activated in several neuronal regions, which could produce sedation, dysphoria-, anxiety- or depression-like behavior that may promote excessive drinking. For examples, after chronic alcohol consumption, there are increases in the KOP-r activity and dynorphin levels observed in the rat central amygdala [D’Addario et al 2013; Kissler et al 2014]. In contrast, nalfurafine at low doses does not produce any dysphoria-, anxiety- or depression-like behavior in rats or mice [Tables 2–5] [Nagase et al 1998; Suzuki et al 2004; Inan et al 2009; Liu et al 2018]. Thereby, nalfurafine could compete to bind the KOP-r with excessive dynorphin, which may be responsible for decreasing excessive alcohol consumption, and reversing the dynorphin-induced dysphoria-, anxiety- or depression-like behavior in alcohol withdrawal.

Nalfurafine is a highly potent agonist, with K_i of 75pM and EC_{50} of 25pM at KOP-r [Seki et al 1999; Wang et al 2005]. In the present study, one concern was that the nalfurafine’s KOP-r agonist activity could produce tolerance after repeated administrations, specifically a gradual reduction in its effect on decreasing alcohol consumption. Indeed, repeated treatments of MSB, a potent KOP-r agonist, showed a blunted effect on reducing alcohol intake after five repeated administrations [Zhou et al 2017]. Using the same multi-dosing regimen, the present study tested the nalfurafine in the IA model and we found that unlike MSB, nalfurafine had a persistent effect on decreasing alcohol consumption after the repeated administrations (Figure 5), indicating that nalfurafine did not show a tolerance after the multi-dosing regimen tested. Consistently, relative to other “classic” KOR agonists, nalfurafine is found to have lower evidence of tolerance to its analgesic effect in animals [Suzuki et al 2004]. Additionally, tolerance to the antipruritic effects of nalfurafine was not observed after chronic treatment in patients with the drug for one year [Kozono et al 2018]. Of interest, nalfurafine has revealed no indications of either physical nor psychological dependence in rodents [Tsuji et al 2000, 2001; Suzuki et al 2004], monkeys [Nakao et al 2016] or humans [Kamimura et al 2017; Kozono et al 2018], along with no evidence of rewarding or reinforcing effects.

In our second main goal, we explored potential synergistic effects of the nalfurafine and naltrexone combination. In rodents, nalfurafine is relatively selective for the KOP-r receptor

in vivo [Schattauer et al 2017; Liu et al 2018]. Given that nalfurafine and naltrexone have clearly different mechanisms, our investigation on the combination of the two drugs could be particularly interesting. Due to their relatively high selectivity for each opioid receptor subtype (nalfurafine for KOP-r and naltrexone for MOP-r) and the low doses used in our experiments, we predicted that the nalfurafine effect on the KOP-r would not be affected by naltrexone's blockade at the MOP-r. Indeed, our results demonstrated that the combination of nalfurafine+naltrexone had a synergistic effect of the individual drugs on decreasing alcohol consumption. The two indications that the combination is more effective and potentially more beneficial than either drug alone are: (1) the effects of these combined, low-dose administrations of nalfurafine+naltrexone on alcohol drinking were 3–4 times greater than those of either drug alone (Figure 2); and (2) the combination showed a longer effect (8 hours) after single administration in both male and female mice than nalfurafine alone (as discussed below) (Figure 4). Finally, the lack of any effect on sucrose or saccharin suggests a specific effect of the combination on alcohol (Table 2).

The mechanistic hypothesis that the MOP-r activation by endogenous beta-endorphin has a different mechanism driving excessive alcohol drinking from the KOP-r-mediated pathway was further examined in the present study. Using the IA model, we previously found that nPE knockout mice lacking central beta-endorphin had lowered alcohol consumption in both sexes, suggesting a decreased rewarding effect of alcohol when central beta-endorphin is deficient [Zhou et al 2018b]. Therefore, we purposely determined here if the activation of KOP-r by nalfurafine could further decrease alcohol intake in nPE knockout mice as a genetic control for the naltrexone effect. As shown in Figure 6, nPE^{-/-} mice displayed a significant decrease in alcohol intake after single administration of nalfurafine at a sub-effective dose, indicating a sensitized effect of nalfurafine after the central POMC/beta-endorphin deficiency. This also suggests that the presence of nalfurafine effects in nPE^{-/-} mice was due to an independent and different pathway or mechanism from that of naltrexone. As the effectiveness of this nalfurafine+naltrexone combination could include multiple neuro-pharmacological mechanisms (at least KOP-r and MOP-r), the combination showed synergistic in decreasing alcohol drinking in the B6 mice. Indeed, neurobiological studies have also provided supportive results, given the multiple actions of alcohol in the brain and that both the KOP-r and MOP-r systems are profoundly altered by chronic alcohol exposure [D'Addario et al 2013; Rácz et al 2013; Kissler et al 2014; Zhou and Kreek 2018]. Alcohol exposure also leads to increased dopamine levels in the nucleus accumbens, leading to rewarding effects, and KOP-r agonists oppose these effects by modulating the dopamine release [Spanagel et al 1990; Lindholm et al 2007], suggesting a potential interaction between dopamine and KOP-r activation by nalfurafine involved in alcohol-related behavior. Naltrexone's actions are mediated through the blockade of MOP-r in the mesolimbic circuitries that may reduce the alcohol rewarding effect (positive reinforcement). Therefore, by aiming at both the KOP-r and MOP-r implicated in alcohol addiction, the combination of nalfurafine and naltrexone is very likely to have enhanced efficacy over the single-receptor approaches. In humans, KOP-r activation is involved in hypothalamic-pituitary-adrenal (HPA) regulation [Ur et al 1997; Schluger et al 1998]. As naltrexone also activates the HPA axis and decreases alcohol craving [O'Malley et al 2002], the combination of nalfurafine and

naltrexone may synergistically modulate the HPA activity, which may also contribute to the enhanced effect of the combinations.

Nalfurafine alone decreased excessive alcohol drinking at 4 hours (Figure 1) with a similar profile to naltrexone alone (Figure S1), a reference compound on the time courses used in our mouse IA model. Of note, we observed a relatively long duration (about 8 hours) of the effect on alcohol drinking behavior when nalfurafine was combined with naltrexone (Figure 4). It is unlikely that naltrexone altered the transport, metabolic stability and bioavailability of nalfurafine, as suggested by previous studies on different metabolism pathways between the two compounds and the drug-drug interactions [Ando et al 2012, 2016]. Of interest, a longer duration of nalfurafine in the nPE knockout mice (about 8 hours) than that in the nPE wildtype (about 4 hours) was also observed (Table S1A, B), further supporting our hypothesis that synergistic effect of nalfurafine+naltrexone combination with longer effects is due to separate and independent neuro-pharmacological mechanisms, including both the MOP-r and KOP-r systems. Though the precise mechanisms are not fully revealed, the new nalfurafine+naltrexone combination with less tolerance and longer duration may have great potential to yield a clinical therapy for treating alcoholism.

In summary, the present study on excessive alcohol drinking in mice constitutes an interesting extension to the anti-addictive properties of nalfurafine observed in cocaine, nicotine or opiate related behaviors [Tsuji et al 2001; Mori et al 2002; Townsend et al 2017]. The finding provides further promising *in vivo* data indicating that nalfurafine, in combination with naltrexone, may offer novel strategies to treat alcoholism, without potential side effects of “classic” KOP-r agonists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement:

NIH AA021970 (YZ), Robertson Therapeutic Discover Fund at the Rockefeller University (YZ), Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (MJK), and NIDA Division of Drug Supply and Analytical Services. Special thanks to Ariel Ben-Ezra for providing her editing corrections on the manuscript.

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Figure 1.

Effects of single administration of nalfurafine (NFF, 10ug/kg) on alcohol intake (A), water intake (B), and preference ratio (C) in male and female mice after 3-week chronic intermittent access drinking. (1) Control groups: males (n=7) and females (n=14) received saline injection (i.p.); and (2) NFF groups: males (n=8) and females (n=14) received one NFF injection (i.p.) 30 min before the drinking test. Alcohol and water intake values were recorded after 4, 8 and 24 hours. * $p < 0.05$ vs. control at the same time point. Data in table are presented as mean+SEM.

Figure 2.

Dose response of single administration of nalfurafine (NFF, 0, 0.3, 1, 3 or 10 $\mu\text{g/kg}$) alone or combined with naltrexone (NTN, 0, 0.3 or 1 mg/kg) on decreasing alcohol intake (A) and alcohol preference ratio (B) in male and female mice after 3-week chronic intermittent access drinking. Data were collected at the first 4-hour recording time on the baseline and the testing day (24 hours later) and are expressed as a percentage of baseline intake to account for the differences in baseline that contribute to variation between experiments ($n=6-14$). * $p<0.05$ or ** $p<0.01$ vs. control (both NFF and NTN at 0 mg/kg); # $p<0.05$ between treatment groups. Data in table are presented as mean+SEM.

Figure 3.

Pretreatment with selective KOP-r antagonist nor-BNI (2.5mg/kg) blocks the effect of single nalfurafine (NFF, 10ug/kg) on decreasing alcohol intake (A) and preference ration (B), but not water intake (C) in male mice (n=6–8). *p<0.05 vs. vehicle control with the same pretreatment. Data in table are presented as mean+SEM.

Figure 4.

Effects of single nalfurafine (NFF) administration at 1ug/kg combined with naltrexone (NTN, 1mg/kg) on alcohol intake (A), water intake (B) and preference ratio (C) in male (left) and female (right) mice (n=6–8) after 3-week chronic intermittent access drinking. Control groups: mice received one saline (i.p.) followed by saline. Test groups: mice received one NFF injection (1ug/kg, i.p.) followed by one NTN injection (1mg/kg, i.p.) before the alcohol drinking test. Alcohol and water intake were recorded after 4, 8 and 24

hours. * $p < 0.05$ vs. control at the same time point. Data in table are presented as mean $\pm$ SEM.

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A. alcohol intake **B. alcohol preference ratio**

Figure 5. Effects of repeated administrations of nalfurafine (10ug/kg) on alcohol intake (A) and preference ratio (B) after 3-week intermittent access drinking in male mice (n=6). * $p < 0.05$ vs. the baseline. Data in table are presented as mean+SEM.

Figure 6.

Genotype differences in the effects of acute nalfurafine (1ug/kg) on alcohol intake (A), preference ratio (B) and water intake (C) after 3-week intermittent access drinking at 4 hours in male (left) and female (right) nPE mice (n=6). In each sex, mice were assigned to one of four groups: (1) nPE+/+ with vehicle; (2) nPE+/+ with 1ug/kg nalfurafine; (3) nPE-/- with vehicle; and (4) nPE-/- with 1ug/kg nalfurafine. Genotype difference: *p<0.05 vs. nPE+/+ mice after the same treatment; Nalfurafine effect: #p<0.05 vs. vehicle control in the same genotype. Data in table are presented as mean+SEM.

Figure 7.

No Effect of single administration of nalfurafine (3 or 10ug/kg) on alcohol intake after 3-week drinking-in-the-dark (DID) in male (left) or female (right) mice (n=8). Data in table are presented as mean+SEM.

Table 1

No effects of single administration of nalfurafine (NFF, 10ug/kg) (**A**), naltrexone (NTN, 3mg/kg) (**B**), or their combination (1ug/kg NFF+1mg/kg NTN) (**C**) on total fluid intake (ml) in male and female mice after 3-week intermittent access drinking. Data in table are presented as mean $\pm$ SEM.

A.	male (n=7-8)		female (n=14)	
time	Vehicle	10ug/kg NFF	Vehicle	10ug/kg NFF
0-4h	1.5 $\pm$ 0.21	1.6 $\pm$ 0.15	1.5 $\pm$ 0.20	1.6 $\pm$ 0.17
5-8h	1.1 $\pm$ 0.16	1.1 $\pm$ 0.21	1.6 $\pm$ 0.21	1.4 $\pm$ 0.22
9-24h	2.9 $\pm$ 0.24	2.9 $\pm$ 0.31	3.2 $\pm$ 0.30	3.3 $\pm$ 0.40

B.	male (n=6-8)		female (n=6-8)	
time	Vehicle	3mg/kg NTN	Vehicle	3mg/kg NTN
0-4h	1.7 $\pm$ 0.32	1.6 $\pm$ 0.31	1.4 $\pm$ 0.25	1.5 $\pm$ 0.30
5-8h	1.3 $\pm$ 0.30	1.4 $\pm$ 0.26	1.7 $\pm$ 0.41	1.4 $\pm$ 0.33
9-24h	3.1 $\pm$ 0.41	3.0 $\pm$ 0.40	3.3 $\pm$ 0.40	3.3 $\pm$ 0.35

C.	male (n=6-8)		female (n=6-8)	
time	Vehicle	1ug/kg NFF+ 1mg/kg NTN	Vehicle	1ug/kg NFF+ 1mg/kg NTN
0-4h	1.3 $\pm$ 0.28	1.3 $\pm$ 0.33	1.7 $\pm$ 0.37	1.4 $\pm$ 0.29
5-8h	1.3 $\pm$ 0.30	1.8 $\pm$ 0.40	1.5 $\pm$ 0.31	1.6 $\pm$ 0.33
9-24h	3.1 $\pm$ 0.44	3.2 $\pm$ 0.59	3.4 $\pm$ 0.40	3.5 $\pm$ 0.47

Table 2

No effect of single administration of nalfurafine (NFF, 10ug/kg) alone or the combination of NFF (1ug/kg) and naltrexone (NTN, 1 mg/kg) on 4% sucrose (A and B) or 0.1% saccharin (C and D) intake, water intake and their preference ratio in male or female mice (n=6–7) at 4-hour time point. Data in table are presented as mean $\pm$ SEM.

A	male		female	
Treatment	Vehicle	10ug/kg NFF	Vehicle	10ug/kg NFF
Sucrose (4%) intake (g/kg)	10.9 $\pm$ 0.72	10.5 $\pm$ 0.93	12.3 $\pm$ 0.95	13.1 $\pm$ 1.2
Water intake (ml)	0.15 $\pm$ 0.08	0.13 $\pm$ 0.03	0.15 $\pm$ 0.05	0.18 $\pm$ 0.11
Preference ratio	0.97 $\pm$ 0.01	0.96 $\pm$ 0.02	0.98 $\pm$ 0.02	0.97 $\pm$ 0.01

B	male		female	
Treatment	Vehicle + Saline	1ug/kg NFF + 1mg/kg NTN	Vehicle + Saline	1ug/kg NFF + 1mg/kg NTN
Sucrose (4%) intake (g/kg)	9.8 $\pm$ 0.98	9.9 $\pm$ 0.92	11.9 $\pm$ 0.91	10.8 $\pm$ 1.0
Water intake (ml)	0.17 $\pm$ 0.06	0.20 $\pm$ 0.09	0.16 $\pm$ 0.07	0.19 $\pm$ 0.10
Preference ratio	0.97 $\pm$ 0.02	0.98 $\pm$ 0.01	0.97 $\pm$ 0.01	0.98 $\pm$ 0.01

C	male		female	
Treatment	Vehicle	10ug/kg NFF	Vehicle	10ug/kg NFF
Saccharin (0.1%) intake (g/kg)	0.13 $\pm$ 0.02	0.14 $\pm$ 0.03	0.18 $\pm$ 0.01	0.17 $\pm$ 0.02
Water intake (ml)	0.18 $\pm$ 0.10	0.21 $\pm$ 0.04	0.14 $\pm$ 0.05	0.15 $\pm$ 0.03
Preference ratio	0.96 $\pm$ 0.04	0.97 $\pm$ 0.02	0.98 $\pm$ 0.01	0.97 $\pm$ 0.02

D	male		female	
Treatment	Vehicle + Saline	1ug/kg NFF + 1mg/kg NTN	Vehicle + Saline	1ug/kg NFF + 1mg/kg NTN
Saccharin (0.1%) intake (g/kg)	0.19 $\pm$ 0.04	0.17 $\pm$ 0.05	0.20 $\pm$ 0.04	0.18 $\pm$ 0.03
Water intake (ml/4h)	0.16 $\pm$ 0.05	0.19 $\pm$ 0.08	0.16 $\pm$ 0.05	0.20 $\pm$ 0.05
Preference ratio	0.97 $\pm$ 0.03	0.98 $\pm$ 0.02	0.98 $\pm$ 0.02	0.97 $\pm$ 0.03

Table 3

No effect of single administration of nalfurafine (10ug/kg) on locomotor activity in alcohol-naïve male and female mice. Locomotor activity is assessed as the number of “crossings,” defined as breaking the light beams at either end of the conditioning chamber. Data in table are presented as mean $\pm$ SEM.

Sex	Male (n = 6)		Female (n = 6)	
Treatment	vehicle	10 ug/kg	vehicle	10 ug/kg
Crossings (30 min)	228 $\pm$ 60	199 $\pm$ 48	268 $\pm$ 55	251 $\pm$ 42

Table 4

No effect of single administration of nalfurafine (10ug/kg) on anxiety-like activity in alcohol-naïve male and female mice. Anxiety-like behavior levels were measured for 5 min by elevated plus maze 30 min after nalfurafine. Data in table are presented as mean $\pm$ SEM.

Sex	Male (n = 7–8)		Female (n = 8)	
	vehicle	10ug/kg	vehicle	10ug/kg
Number of open arm entries	11 $\pm$ 1.0	8.9 $\pm$ 1.2	10 $\pm$ 1.5	9.2 $\pm$ 1.1
Time spent in open arms (sec)	38 $\pm$ 4.1	40 $\pm$ 4.9	42 $\pm$ 4.5	39 $\pm$ 7.1

Table 5

No effect of single nalfurafine (10ug/kg) on conditioned place aversion (CPA) in alcohol-naïve male and female mice. The time spent in either the nalfurafine-paired compartment or the vehicle (saline)-paired compartment was recorded for 30 min in both the preconditioning session and CPA test for 30 min. Data in table are presented as mean $\pm$ SEM.

Sex	Male (n = 6)		Female (n = 6)	
Treatment	vehicle	10ug/kg	vehicle	10ug/kg
Preconditioning: time spent in chambers (sec)	462 $\pm$ 49	477 $\pm$ 60	431 $\pm$ 55	429 $\pm$ 61
CPA test: time spent in chambers (sec)	438 $\pm$ 52	420 $\pm$ 46	452 $\pm$ 45	449 $\pm$ 69