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TITLE PAGE

Dual efficacy of delta opioid receptor selective ligands for ethanol drinking and anxiety.

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Non-standard abbreviations: DOR, delta opioid receptor; DOR1, delta opioid receptor subtype 1; DOR2, delta opioid receptor subtype 2; MOR, mu opioid receptor; KO, knockout; AD, anxiety disorder, TAN-67 (SB-205607) (2-methyl-4a alpha-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12a alpha-octahydro-quinolino[2,3,30g]isoquinoline; SNC80, (+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide; DZ, diazepam (7-Chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2(1H)-one); NTX, naltrexone (17-(cyclopropylmethyl)-4,5α-epoxy- 3,14-dihydroxymorphinan-6-one); NTB, naltriben (17-(cyclopropylmethyl)-6, 7-didehydro-3, 14β-dihydroxy-4, 5α-epoxy-6, 7-2,3'-benzo furanomorphinan); SSRIs, selective serotonin reuptake inhibitors ; BZDs, benzodiazepines

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Abstract

Alcoholism and anxiety disorders have a huge impact on society, and afflict 17.6 million and 40 million people in the USA, respectively. A strong co-morbidity exists between alcoholism and anxiety disorders. Indeed, alcohol withdrawal-induced anxiety is a primary contributing factor for relapse, and anxiolytics are a common adjuvant therapy prescribed for treatment-seeking alcoholics. It is thought that the use of alcohol to self-medicate and relieve anxiety contributes to the development of addiction. Treatment for anxiety disorders and alcoholism exist but are not universally effective. The delta opioid receptor (DOR) plays a role in both alcohol consumption and anxiety making it a very interesting clinical target. Two pharmacologically distinct DORs have been described: DOR1 and DOR2. We find here that the relative specificity of DOR agonists for DOR1 or DOR2, can greatly impact the effects they exert on ethanol consumption and anxiety. The DOR1 agonist TAN-67, while not effective in decreasing anxiety-like behavior in naïve mice, has anxiolytic-like properties in ethanol-withdrawn mice. In contrast, a less subtype selective agonist, SNC80, while also reducing anxiety-like behavior, increases ethanol consumption. Additionally, we found that the conical anxiolytic diazepam is a less effective anxiolytic in ethanol-withdrawn mice than in naïve mice. Together, our findings suggest that selective DOR agonists can decrease anxiety-like behavior, and are more effective than diazepam at reducing ethanol consumption. We believe the dual efficacy of DOR1 agonists makes these receptors an interesting therapeutic target for treatment-seeking alcoholics.

Introduction

Currently three drugs have been approved by the federal drug administration to treat alcoholism: Revia (Naltrexone [NTX], a non-selective opioid antagonist), Campral (acamprosate, a NMDA receptor antagonist), and Antabuse (Disulfiram, an inhibitor of acetylaldehyde dehydrogenase). While these drugs can be efficacious in reducing ethanol consumption, all have clinical limitations and suffer from compliance issues (Pettinati et al., 2000; Buonopane and Petrakis, 2005; Anton et al., 2006; Swift, 2007; Garbutt, 2009; Mitchell et al., 2009). One of the drugs currently in clinical trials to treat alcoholism is the neurokinin (NK1) receptor antagonist LY686017. It is thought that the efficacy of this drug is due, at least in part, to the reduction of anxiety and craving in anxious alcohol-dependent subjects (George et al., 2008; Heilig et al., 2009). According to the National Institute of Mental Health, approximately 40 million US American adults suffer from an anxiety disorder [AD]. The individual and societal cost associated with ADs is extremely high (Wittchen, 2002; Hoffman et al., 2008). Importantly, people suffering from ADs are more susceptible to substance abuse (Wittchen, 2002; Hoffman et al., 2008). Selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines (BZDs) are the two classes of drug most commonly used to treat ADs (Cloos and Ferreira, 2009). SSRIs are generally well-tolerated; however, they take several weeks to take effect, and each SSRI is only effective in a minority of patients (Simon, 2001), who cannot be identified prior to initiating treatment; (Tiwari et al., 2009). BZDs are commonly prescribed to control anxiety “attacks;” however, they themselves are intoxicating and habit-forming (Isbister et al., 2004; O'Brien C, 2005). Importantly, BZDs can increase the palatability of ethanol and increase alcohol consumption (Soderpalm and Hansen, 1998). Thus, there is

significant need for novel targets and treatments for ADs, especially for anxiety that is co-morbid with alcoholism.

One target that is linked to anxiety is the delta opioid receptor (DOR). Disruption of the gene encoding DOR or its endogenous ligand produces an anxious-like phenotype in mice (Filliol et al., 2000; Ragnauth et al., 2001; Roberts et al., 2001), suggesting that DOR agonists could be anxiolytic. The DOR is a particularly interesting target for anxiety, because it has also been found to regulate ethanol consumption. In some cases mice disrupted for DOR show enhanced ethanol consumption when alcohol naïve (van Rijn and Whistler, 2009), but DOR knock out mice also show enhanced drinking and preference for alcohol once they have been drinking for some time (Roberts et al., 2001). Importantly, two DOR subtypes (DOR1 and DOR2) can be distinguished *in vivo* (Mattia et al., 1991; Zaki et al., 1996), and recently we found that these subtypes have opposing effects with regard to ethanol intake (van Rijn and Whistler, 2009).

As mentioned above, there is significant co-morbidity between anxiety and alcohol abuse, and anxiety and stress are risk factors that predispose an individual to both the primary development of alcoholism as well as relapse in withdrawing and abstinent patients. Individuals abstaining from ethanol may experience increased levels of anxiety, especially within the first 24-48 hours after their last drink, a phenomenon generally known as the alcohol withdrawal syndrome. This is often treated with benzodiazepines (McKeon et al., 2008). Stress-related-anxiety-induced relapse can also be modeled in pre-clinical animal models, as rats subjected to a foot shock paradigm, for example, show increased relapse to alcohol (Le et al., 1999; Liu and Weiss, 2003). As would be expected if activity at the DOR were anxiolytic, rats that express more functional DORs show less

anxiety-like behaviors and drink less ethanol than rats with fewer functional receptors (Margolis et al., 2008), and DOR ligands reduce ethanol seeking in withdrawn rats (Ciccocioppo et al., 2002; Marinelli et al., 2009).

Here, we investigate the ability of DOR selective agonists to decrease ethanol consumption and both anxiety- and ethanol withdrawal-induced anxiety-like behaviors compared with drugs (NTX and diazepam) currently available to treat these disorders. We find that the selective DOR agonist SNC80 reduces anxiety-like behaviors, but also increases ethanol consumption. In comparison, the DOR1-selective agonist TAN-67, which we had previously shown to decrease ethanol intake (van Rijn and Whistler, 2009), has no effect on anxiety-like behavior in alcohol naïve mice. Interestingly however, we observe that TAN-67 does reduce ethanol withdrawal-induced anxiety-like behaviors. In contrast, we find that two drugs, commonly used in the treatment of alcoholism (NTX) or anxiety (diazepam), may induce anxiogenic-like behavior and have limited ability to decrease ethanol consumption, respectively. Therefore, DOR1 subtype-selective drugs may potentially have an improved ability to simultaneously reduce ethanol intake and anxiety associated with ethanol abstinence than the currently available therapeutics.

Methods

Animals and housing. Wild-type and DOR knockout C57BL/6 mice (male, 20-25g, Taconic) were housed (maximally 5 per cage) in ventilated plexiglass cages at ambient temperature (21ºC) in a room maintained on a 12L:12D cycle (lights on at 8.00, lights of at 20.00). Food and water was provided ad libitum. The mice were given one week to acclimatize before the start of the experiments. All animal procedures were pre-approved by the Gallo Center Institutional Animal Care and Use Committee, performed in our AAALAC certified facility, and were in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice were not deprived of food or water at any time.

Chronic ethanol exposure. The limited access two-bottle choice paradigm was performed as previously described (van Rijn and Whistler, 2009). In short, for the ethanol exposure, mice were individually housed in ventilated plexiglass cages at ambient temperature (21ºC) in a room maintained on a reversed 12L:12D cycle (lights off at 10.00, lights on at 22.00). Food and water was provided ad libitum. The mice were given one week to acclimatize to the individual housing conditions and reverse light cycle before the start of the experiments. A two-bottle, limited access (4 hours/day), drinking paradigm was employed for one week (5 days). During the limited drinking phase mice had access to water and either a 10% ethanol solution or a 2% sucrose solution.

Anxiety-like behavior measurements

Elevated plus maze. The elevated plus maze consisted of two closed arms and two open arms arranged perpendicular to one another. The plus-maze was made of wood, painted white and elevated 41 cm above the floor (arm length 70 cm, width 9 cm, and height 12 cm). No rim was present surrounding the open arm. During the five minute trial, the behavior of the mouse was recorded by a camera positioned above the maze, in the absence of laboratory personnel. Each mouse was placed in the center of the maze (9 cm x 9 cm) facing a closed arm. The surface of the maze was cleaned with disinfectant and dried before the next mouse was tested. Light intensity on the plus maze was 460 lux. The variables measured included the total number of entries into the closed and open arms and the total time spent in each region. An entry was defined as the mouse placing two paws within the boundaries of the arm. An increase in the number of entries and time spent in the open arms is indicative of an anxiolytic-like response.

Dark-Light transition box. The dark-light apparatus was made up of an automated activity monitor with a dark box insert (ENV-510, Med associates), to create an equally spaced light and dark compartment (24 cm x 28 cm x 25 cm). The entire apparatus was positioned in a sound-attenuating chamber. The light side was illuminated to a degree of 100 lux, compared to 5 lux in the dark side. Each animal was placed facing the entrance of the dark area and their behavior was recorded for five minutes. The dark-light transition box was cleaned with disinfectant and dried before the next mouse was tested. A photobeam-based tracking system was used to track the movement and locomotor activity of the mice within the test box and to calculate the time spent in each area and the number of entries into each area. Anxiolytic-like effects were indicated by increased time spent in the illuminated compartment.

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Ethanol-withdrawal-induced anxiety. To study ethanol withdrawal-induced anxiety-like behaviors, mice were trained in the limited access two-bottle choice paradigm for 5 days. On day 6 (24 hour after the last access to ethanol or sucrose), anxiety levels in the withdrawn mice were tested using the elevated plus maze and the dark-light transition box.

Data analysis. Baseline values for the ethanol drinking studies were determined by taking the average of the consumption over the three days prior to injection. Statistical analysis was performed using Prism software (GraphPad, San Diego, CA). Significance was determined by means of student t-test, one-way ANOVA or two-way ANOVA (repeated measures). A post-hoc Newman-Keuls (one-way ANOVA) or Bonferroni (two-way ANOVA) test was used when a significant overall effect was found ($p<0.05$). (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Drugs. Ethanol solutions were prepared in tap water using 95% (vol/vol) ethanol (Gold Shield Chemical Co., Hayward, CA, USA) (±)-SB205607 dihydrobromide (TAN-67, 25 mg/kg) was purchased from Tocris (MO, USA). Sucrose, naltrexone (NTX, 1 mg/kg), SNC80 (20 mg/kg), and diazepam (1 & 3 mg/kg) were purchased from Sigma-Aldrich (MO, USA). All compounds were dissolved in saline. Diazepam was suspended in solution with 0.06% Tween 80. This concentration of Tween has no effect on behavior (Supplemental Figure 1A and B) and, therefore, we used saline as vehicle in all our experiments to allow comparison across all treatment groups. All drugs were prepared

immediately prior to injection and were administered sub-cutaneously (s.c.). All drugs were administered 30 minutes before the beginning of each experiment.

Results

Delta opioid receptors affect alcohol intake and anxiety-like behaviors. We have previously shown that C57BL/6 mice with a knockout of the DOR gene (DOR KO) consume more ethanol than wild-type mice (van Rijn and Whistler, 2009). We find that these C57BL/6 mice also show an anxiogenic phenotype, as measured by their behavior on an elevated plus maze (Figure 1A-C) and dark-light transition box (Figure 1D). The WT mice spent significantly more time in the open arm (p = .014) of the elevated plus maze (Figure 1A) and the light side (p = .013) of the dark-light box (Figure 1D) compared to DOR) mice. In addition, the DOR KO mice also made significantly (p = .0001) fewer entries in the open arm of the elevated plus maze compared to WT mice (Figure 1B). The fewer entries in DOR KO was not due to a generalized decreased locomotor activity as DOR KO mice were somewhat hyper-locomotive (p = .026) compared to WT mice (Figure 1E). The two genotypes showed no difference in the amount of time the mice spent in the center of the elevated plus maze (Figure 1C).

DOR selective ligands can either increase or decrease ethanol intake depending on the subtype targeted. We have previously reported that agonists selective for DOR1 (TAN-67) and antagonists selective for DOR2 (Naltriben, (NTB)) decrease ethanol consumption in mice ((van Rijn and Whistler, 2009), and see Figure 2A and 2B), suggesting that DOR1 and DOR2 receptors have opposing effects on ethanol consumption. Here we found that the DOR agonist SNC80 significantly increased ethanol consumption [$F(2,24) = 20.48$, $p<0.0001$] (Figure 2A, B). Thus, the

pharmacological specificity of the DOR1 agonist TAN-67 was critical for its ability to reduce ethanol consumption. Naltrexone (NTX) the current FDA-approved drug used in the treatment of alcoholism can also decrease ethanol consumption in mice ((van Rijn and Whistler, 2009), and see Figure 2C and 2D), but also decreased water consumption and therefore did not effect preference (Figure 2D). In addition, the ability of NTX to antagonize all three opioid receptors (Raynor et al., 1994) may contribute to the side effects of this drug. The benzodiazepine diazepam had no significant effect on either ethanol consumption [$F(2,24) = 28.22$, $p<0.0001$] or preference [$F(2,24) = 1.78$, $p = .19$], although there was a trend towards an increase in alcohol consumption (Figure 2C and 2D), consistent with previous reports that benzodiazepines can increase the palatability of ethanol (Soderpalm and Hansen, 1998). Thus, among these four drugs, we tested, the DOR1-selective agonist TAN-67, the DOR-selective agonist SNC80, the opioid receptor antagonist NTX and the benzodiazepine diazepam, only the DOR1 agonist TAN-67 reduced both ethanol consumption and preference [$F(2,24) = 6.21$, $p = .0067$].

The DOR-selective agonist SNC80, but not the DOR1-selective agonist TAN-67 reduces anxiety-like behavior in naïve mice. Since disruption of DORs increases anxiety-like behavior (Figure 1), we next examined whether DOR agonists could decrease anxiety, and whether this effect was subtype specific. Both the DOR-selective agonist SNC80 [$F(2,41) = 5.46$, $p = .0079$] and our control anxiolytic diazepam [$F(2,39)$, $p = .023$] produced anxiolytic-like properties in the dark-light transition box (Figure 3A and 3C), significantly increasing time spent in the light side. Neither SNC80 nor diazepam significantly [$F(2, 42) = 0.52$, $p = .60$] increased the time mice spent in the

open arm of the elevated plus maze (Figure 3D). However, this is likely because even in the absence of drug alcohol naïve C57BL/6 mice, spent nearly 30% of their time in the open arm. While the amount of time spent in the open arm is relatively high this finding in not unusual for this strain of mice (Griebel et al., 2000; Lepicard et al., 2000; Carola et al., 2002; Lalonde and Strazielle, 2008). Additionally, it is not uncommon for 1 mg/kg diazepam to fail to reduce anxiety in C57Bl/6 mice in the elevated plus maze paradigm (Griebel et al., 2000; Lepicard et al., 2000). Interestingly, we found that mice treated with NTX spent significantly [$F(2,41) = 4.19$, $p = 0.023$] less time in the open arm of the elevated plus maze (Figure 3D). This suggests that NTX may elicit some anxiety like behavior even in alcohol naïve mice, which is in agreement with previous reports that have shown that in some cases NTX can increase anxiety (King et al., 1997; Maremmani et al., 1998; Kozak et al., 2007). Thus, we find the light dark transition box appears to be the more sensitive assay for detecting anxiolytic-like effects while the elevated plus maze appears to be more sensitive for detecting anxiogenic-like effects, at least for C57BL/6 mice.

C57BL/6 mice display ethanol but not sucrose withdrawal-induced anxiety-like behaviors. One of the many problems treatment seeking abstinent alcoholics encounter is an increased level of anxiety (McKeon et al., 2008). In rodents, forced ethanol exposure via bolus intra peritoneal injection of ethanol or use of an ethanol vapor chamber has been shown to produce anxiety-like behaviors after withdrawal (Kliethermes, 2005). Here we developed a paradigm to measure anxiety-like behavior following withdrawal from voluntary ethanol consumption. As above, mice were given a choice of water and 10%

ethanol (or 2% sucrose as a controlled "preferred" substance) for 4 hours a day for 5 days. After a five day period both 10% ethanol and 2% sucrose are preferred over water to a similar extent (Figure 4A). Mice were then examined for anxiety-like behavior, 24 hours after their last ethanol or sucrose exposure. Ethanol withdrawn mice showed a significant reduction in time spent in the open arm of the elevated plus maze compared to naïve mice or sucrose withdrawn mice [$F(2,28) = 8.02$, $p = .0018$] (Figure 4B) indicative on increased anxiety. In the dark light transition paradigm we observe an anxiogenic trend between the naïve and ethanol withdrawn mice. Whereas in comparison to sucrose withdrawn mice, we find that ethanol withdrawn mice do in fact spend significantly less time in the light side of the dark-light box [$F(2,31) = 4.46$, $p = .020$] (Figure 4C).

The changes in anxiety-like behavior were unlikely due to changes in general locomotion, as neither ethanol nor sucrose withdrawal had a significant effect on locomotor activity [$F(2,31) = 1.14$, p=.33] (Figure 4D).

DOR selective agonists attenuate ethanol withdrawal induced anxiety-like behavior. We next examined whether DOR-selective agonists could decrease ethanol-withdrawal-induced anxiety-like behavior. We found that SNC80 significantly reduced withdrawal-induced anxiety-like behavior in both the dark-light transition box [$F(3,38) = 5.80$, $p = .002$] (Figure 5A) and the elevated plus maze [$F(3,36) = 8.36$, $p = .0002$] (Figure 5B). Importantly, although the DOR-1 selective agonist TAN-67 was ineffective at reducing anxiety-like behavior in alcohol naïve mice, it did reduce anxiety like behavior in ethanol withdrawn mice. Specifically, in ethanol withdrawn mice, TAN-67 significantly

increased the amount of time spent in the light side of the dark-light transition box (Figure 5A). In addition, ethanol withdrawn mice given TAN-67 no longer spend significantly less time in the open arm than ethanol naïve mice (Figure 5B). Both NTX and diazepam significantly [$F(3,39) = 8.\ 04$, $p = .0003$] increased time spent in the light side of the of the dark-light transition box (Figure 5C). However, only NTX, but not diazepam significantly [$F(3,36) = 9.09$, $p = .0001$] increased the time spent in the open arm of the elevated plus maze (Figure 5D). Benzodiazepines and ethanol both allosterically activate $GABA_A$ receptors. Thus, the reduction in efficacy of diazepam in the ethanol withdrawn mice could reflect "cross tolerance" at this target as a function of decreased number or function of $GABA_A$-receptors as a consequence of alcohol exposure (Sanna et al., 2003). To examine this possibility, we tested whether a higher dose of could overcome the apparent tolerance. However, 3 mg/kg, diazepam produced significant sedative effects, immobilizing mice (Supplemental Figure 1C).

Discussion

Here we show that some but not all DOR subtype selective ligands show dual efficacy at reducing ethanol consumption and ethanol withdrawal induced anxiety. We and others have previously reported that DOR KO mice consume more ethanol and show increased anxiety-like behavior than wild-type mice (Roberts et al., 2001; van Rijn and Whistler, 2009) (Filliol et al., 2000). Together these studies suggest that DORs play a role in both anxiety and drinking behavior. Anxiety is a key risk factor for relapse in human alcoholics, which has led to the use of anxiolytics as adjunct therapy in the treatment of alcoholism. However, here we found that anxiolytics, including the benzodiazepine diazepam and the DOR-selective agonist SNC80, while reducing anxiety like behaviors, increase rather than decrease drinking. Importantly, we found that the DOR1-selective agonist TAN-67, which showed no effect on anxiety like behaviors in alcohol naïve mice, reduced both drinking and alcohol withdrawal induced anxiety.

We hypothesize that the diversity in the effectiveness of distinct opioid drugs for ethanol consumption and anxiety, can be attributed to the existence of multiple receptor subtypes that differentially effect behavior. Two pharmacologically distinct DOR subtypes have been described *in vivo*. Intriguingly, ligands selective for DOR1 or DOR2 have opposing effects on alcohol consumption (van Rijn and Whistler, 2009). Thus, the existence of two DOR subtypes with opposing effects on drinking could explain why results from DOR KO mice do not always directly correlate with those seen with DOR subtype-selective ligands.

Our finding that the DOR-selective agonist SNC80 reduces the expression of anxiety-like behaviors in mice is in agreement with previously published work in rats, showing

SNC80 decreases anxiety-like behaviors (Saitoh et al., 2004), and that the DOR selective antagonist naltrindole (Perrine et al., 2006) and the DOR2-subtype selective antagonist naltriben produce an anxiogenic-like effect in the elevated plus maze in rats (Saitoh et al., 2005). However, although it decreases anxiety, SNC80 increases alcohol consumption. Thus, it appears that decreasing anxiety *per se* does not lead to a decrease in ethanol consumption. Importantly, we found that, unlike SNC80 (and diazepam), the DOR1 agonist TAN-67 decreased ethanol consumption. Although TAN-67 was not anxiolytic in alcohol naïve mice, it eliminated alcohol-withdrawal induced anxiety. Thus TAN-67 is distinguished from SNC80 (and diazepam) in that it reduces rather than enhances drinking and has a selective effect on alcohol-withdrawal induced anxiety. SNC80 and TAN-67 are both highly selective for DOR over MOR (Knapp et al., 1995; Knapp et al., 1996). However, the dextrorotary enantiomer of TAN-67 has been shown to interact with spinal nociceptin/orphanin FQ receptor with nanomolar affinity (Kamei et al., 1999). Further studies are required to determine to what degree, if any, the racemic mixture of TAN-67 or the dextrorotary enantiomer affect ethanol consumption and anxiety through the nociceptin receptor. Our finding that TAN-67 shifts from being ineffective in reducing anxiety-like behavior in naïve mice to having anxiolytic properties in ethanol withdrawn mice, corresponds to findings that DORs may be upregulated after chronic stress and ethanol consumption (Commons, 2003; Margolis et al., 2008). Moreover, we have previously shown that removal of DORs eliminates the effects of TAN-67 on ethanol consumption entirely (van Rijn and Whistler, 2009).

Alcohol dependent individuals, who abstain from consuming ethanol, may experience several withdrawal symptoms, including anxiety, deliriums and potentially life

threatening seizures 24-48 hours after their last drink. While, these symptoms may be mild in occurrence in general, the percentage of people reporting these symptoms increases in the subpopulation of heavy drinkers undergoing detoxification procedures. Benzodiazepines are a common treatment of alcohol withdrawal symptoms (McKeon et al., 2008). Several rodent models exist that can reproduce the ethanol withdrawal-induced anxiety observed in humans. In general, the methods used to induce ethanol dependence require forced exposure to the ethanol, such as ethanol containing liquid diets, ethanol vapor chambers and intra peritoneal ethanol injections (Kliethermes, 2005). The forced nature of these methods however does not reflect human behavior and may further complicate any extrapolation of data obtained in mice to the human situation. Another issue in these rodent models is that withdrawn animals often have a reduction in their locomotor activity, which could be mistaken as a sign of anxiety-like behavior (Kliethermes, 2005). We find that, in a model of voluntary ethanol consumption, ethanol- but not sucrose-withdrawal increases the expression of anxiety-like behavior. Importantly, the locomotor activity of these mice is not affected. Therefore, we believe this paradigm may be a suitable model to study ethanol withdrawal-induced anxiety-like behavior. Most importantly, using this model we found that both SNC80 and TAN-67 are effective in reducing ethanol withdrawal-induced anxiety-like behavior and are, in fact, more effective than diazepam.

The ability of TAN-67 to decrease ethanol withdrawal induced anxiety-like behavior is surprising as TAN-67 does not affect anxiety-like behavior in naïve mice. DOR expression has been shown to change under influence of chronic stressors, such as inflammation (Cahill et al., 2003), and chronic exposure to morphine (Cahill et al., 2001),

ethanol (Margolis et al., 2008) and stress (Commons, 2003). We hypothesize that drinking and/or withdrawal from ethanol, may change the number of functional DORs, in particular DOR1s. An increase in DOR function could explain our finding that both TAN-67 and SNC80 become more effective in the ethanol withdrawn mice. In particular, we would expect chronic ethanol to upregulate the number or function of DOR1s in circuits or brain regions that control anxiety. This hypothesis is supported by recent findings, that chronic ethanol exposure recruited functional DORs in the central nucleus of the amygdala (Bie et al., 2009). Intriguingly, a recent study showed that DORs are involved in the function of benzodiazepines (Primeaux et al., 2006). Thus a change in the number of functional DORs, induced by heavy ethanol consumption and stress, could thereby also possibly affect the efficacy of benzodiazepines.

In conclusion, we show that the current drugs available to combat alcoholism in the human population while effective at controlling individual aspects of the disease none are ideally suited to treat both consumption and withdrawal induced anxiety. Diazepam reduces anxiety-like behavior in naïve mice but increases ethanol palatability and consumption and loses efficacy as an anxiolytic after drinking. Conversely, NTX can reduce ethanol consumption, but it suffers from side effects such as dysphoria and increased pain sensitivity and may increase anxiety. Here we show that drugs that selectively target DOR subtypes could show promise as “dual efficacy” drugs to reduce both ethanol consumption and withdrawal induced anxiety. The choice of DOR subtype will be key to the success of this strategy as the DOR1-selective drug TAN-67 decreased both alcohol withdrawal-induced anxiety and consumption while the DOR agonist SNC80 decreased anxiety but increased consumption. In short, we believe that selectively

targeting DOR1s with agonist drugs could be a promising new strategy to reduce ethanol-withdrawal induced anxiety, without increasing the drive for alcohol consumption.

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Footnotes

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Legends for Figures

Figure 1: Delta opioid receptor knockout (DOR KO) show a higher degree of anxiety-like behavior compared to wild-type C57BL/6 mice. Anxiety-like behaviors were measured in wild-type and DOR KO C57BL/6 mice using the elevated plus maze (A-C) and dark-light transition box (D). For the elevated plus maze relative time spent (A) in the open arms and center was measured as well as the number of entries into the open arm (C) for 5 minutes. For the dark-light transition box, relative time spent in the light chambers (D) was measured for 5 minutes. Locomotor activity (distance traveled) was assessed in the dark-light box (E). The number of animals used in each group is indicated in each bar of the histogram. (*p<0.05; ***p<0.001).

Figure 2: Delta opioid receptor subtypes selective agonists have opposing actions in ethanol consumption. Wild-type C57BL/6 mice (n=9), trained to drink in a limited access two-bottle choice paradigm, were injected s.c. with saline, 25 mg/kg of the DOR1 agonist TAN-67, 20 mg/kg of the DOR agonist SNC80 (A and B), 1.5 mg/kg of the non selective opioid antagonist NTX or 1 mg/kg of the benzodiazepine diazepam (C and D). Thirty minutes after injection ethanol and water consumption were measured over a four hour period. Ethanol preference = ethanol consumption/(ethanol consumption + water consumption). (**p<0.01; ***p<0.001).

Figure 3: The DOR selective agonist SNC80 decreases anxiety-like behavior in naïve mice. A, Naïve wild-type C57BL/6 mice were injected s.c. with saline, 25 mg/kg of the

DOR1 agonist TAN-67, 20 mg/kg of the DOR agonist SNC80 (A and B), 1.5 mg/kg of the non selective opioid antagonist NTX or 1 mg/kg of the benzodiazepine diazepam (C and D). Thirty minutes after injection anxiety-like behavior was measured using the dark-light transition box (A and C) and the elevated plus maze (B and D). For the dark-light transition box relative time spent in the light chambers was measured for 5 minutes. For the elevated plus maze relative time spent in the open arms was measured for 5 minutes. The number of animals used in each group is indicated in each bar of the histogram. (*p<0.05; **p<0.01).

Figure 4: Mice trained to voluntarily consume ethanol display ethanol withdrawal induced anxiety-like behavior. C57BL/6 mice (n=9) were trained to consume either ethanol or sucrose (A, see materials and methods). Anxiety-like behavior was measured using the elevated plus maze (B) and dark-light transition box (C). For the elevated plus maze relative time spent in the open arms was measured for 5 minutes. For the dark-light transition box relative time spent in the light chambers was measured for 5 minutes. Locomotor activity (distance traveled) was assessed in the dark-light box (D). The number of animals used in each group is indicated in each bar of the histogram (*p<0.05; **p<0.01).

Figure 5: Delta opioid receptor selective agonists can abolish ethanol-withdrawal induced-anxiety. Ethanol withdrawn (24h after last exposure) C57BL/6 mice, were injected s.c. with saline, 25 mg/kg of the DOR1 agonist TAN-67, 20 mg/kg of the DOR agonist SNC80 (A and B), 1.5 mg/kg of the non selective opioid antagonist NTX or 1

mg/kg of the benzodiazepine diazepam (C and D). Thirty minutes after injection anxiety-like behavior was measured using the dark-light transition box (A and C) and the elevated plus maze (B and D). For the dark-light transition box relative time spent in the light chambers was measured for 5 minutes. For the elevated plus maze relative time spent in the open arms was measured for 5 minutes. The number of animals used in each group is indicated in each bar of the histogram (*p<0.05; **p<0.01; ***p<0.001 [*vs* ethanol]; †p<0.05 [*vs* naive]).

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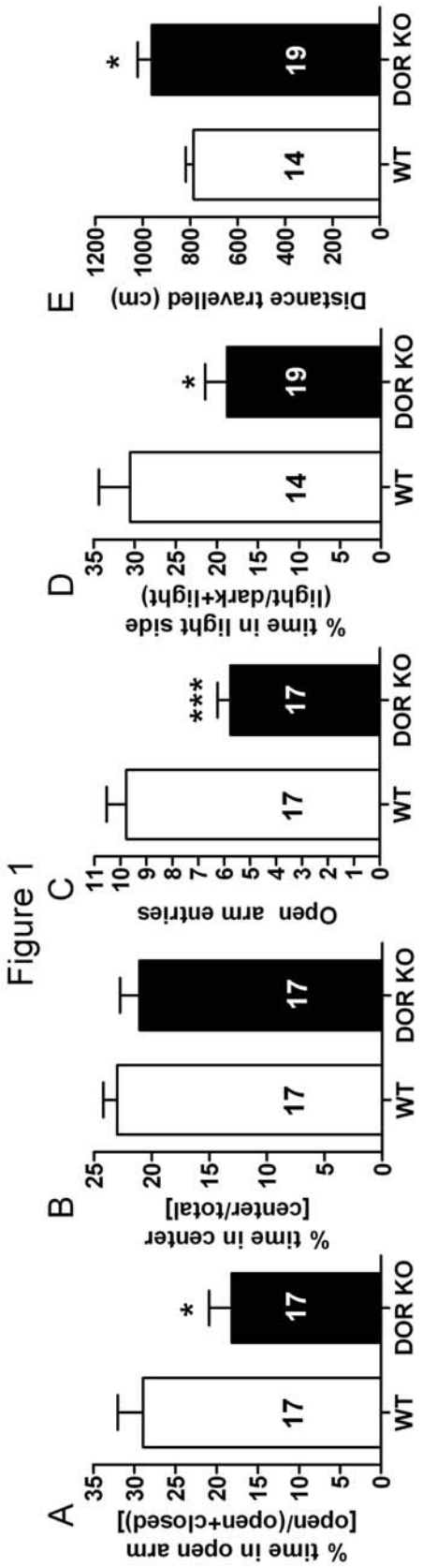

Figure 1

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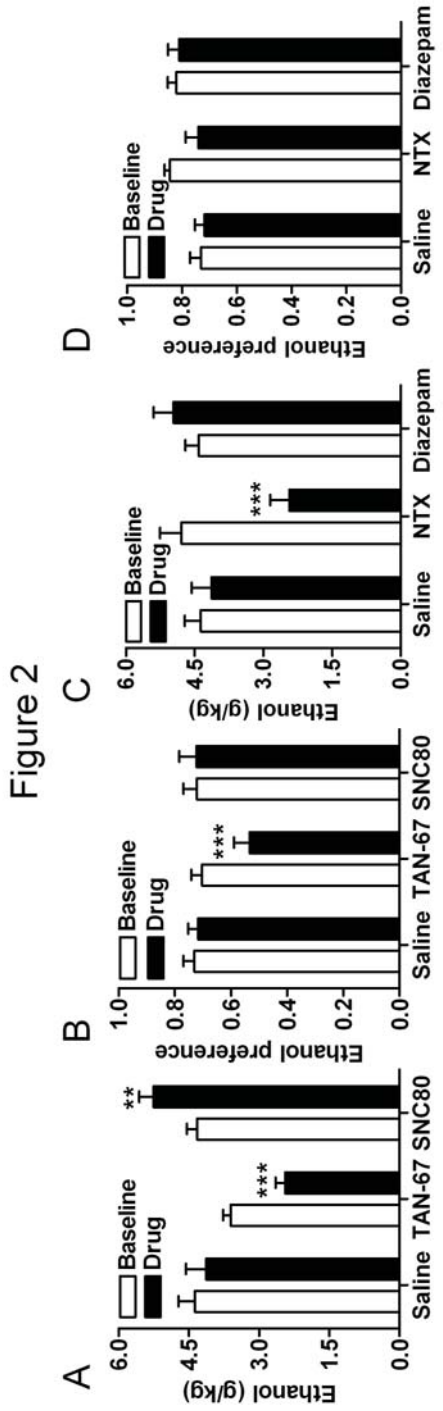
Figure 2



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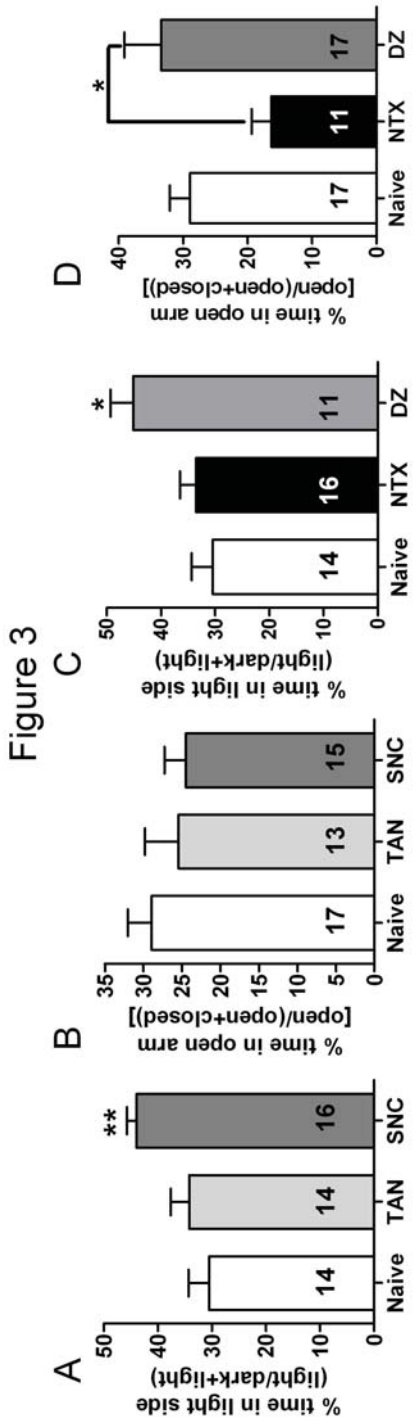

Figure 3

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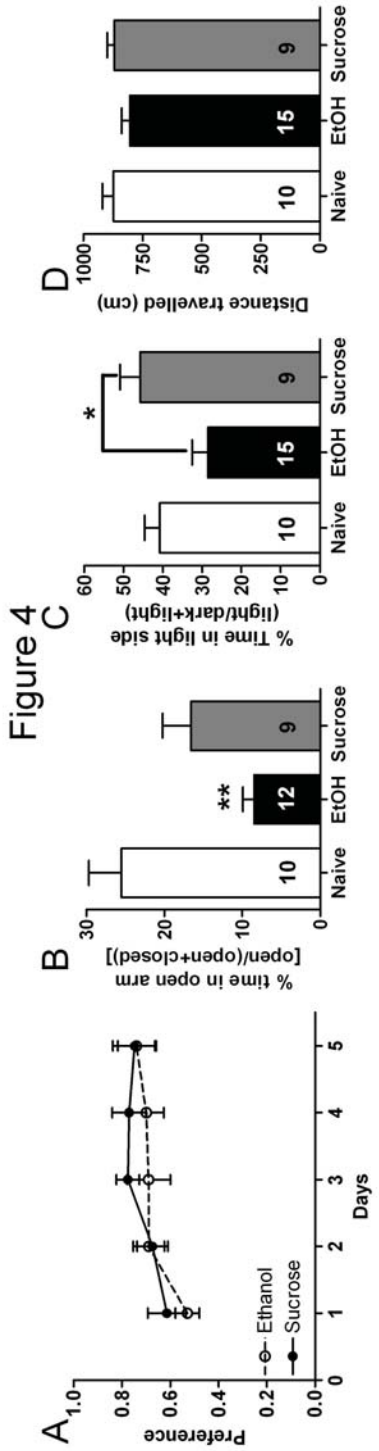

Figure 4

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