

Affective Cue-Induced Escalation of Alcohol Self-Administration and Increased 22-kHz Ultrasonic Vocalizations during Alcohol Withdrawal: Role of Kappa-Opioid Receptors

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Negative affect promotes dysregulated alcohol consumption in non-dependent and alcohol-dependent animals, and cues associated with negative affective states induce withdrawal-like symptoms in rats. This study was designed to test the hypotheses that: (1) the kappa-opioid receptor (KOR) system mediates phenotypes related to alcohol withdrawal and withdrawal-like negative affective states and (2) cues associated with negative affective states would result in dysregulated alcohol consumption when subsequently presented alone. To accomplish these goals, intracerebroventricular infusion of the KOR antagonist nor-binaltorphimine (nor-BNI) was assessed for the ability to attenuate the increase in 22-kHz ultrasonic vocalizations (USVs) associated with alcohol withdrawal and KOR activation in adult male wistar rats. Furthermore, cues associated with a KOR agonist-induced negative affective state were assessed for the ability to dysregulate alcohol consumption and the efficacy of intracerebroventricular KOR antagonism to reduce such dysregulation was evaluated. KOR antagonism blocked the increased number of 22-kHz USVs observed during acute alcohol withdrawal and a KOR agonist (U50,488) resulted in a nor-BNI reversible increase in 22-kHz USVs (mimicking an alcohol-dependent state). Additionally, cues associated with negative affective states resulted in escalated alcohol self-administration, an effect that was nor-BNI sensitive. Taken together, this study implicates negative affective states induced by both alcohol withdrawal and conditioned stimuli as being produced, in part, by activity of the DYN/KOR system.

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INTRODUCTION

Alcoholism is a major problem in the United States and abroad, with an estimated 7.0% of the US population 12 or older dependent on alcohol (Substance Abuse and Mental Health Services Administration, 2010). Recent research suggests early alcohol use as being a significant predictor of future licit and illicit drug use (Kirby and Barry, 2012), and, furthermore, that alcohol is the most harmful of all abused drugs (Nutt *et al*, 2010). Alcohol use disorders (AUDs; comprised of alcohol abuse and alcohol depen-

dence) are commonly comorbid with affective disorders such as depression (Boden and Fergusson, 2011) and negative affective states that are present during withdrawal can promote excessive alcohol use (Koob and Le Moal, 1997). However, there are currently no pharmacotherapies for the treatment of AUDs that focus on this aspect of dysregulated alcohol consumption.

Negative affect has been implicated in the increased consumption of alcohol in non-dependent (Liu and Weiss, 2002; Sperling *et al*, 2010) and dependent animals (Liu and Weiss, 2002). Foot-shock stress has been shown to facilitate conditioned place preferences for alcohol and operant alcohol self-administration (Sperling *et al*, 2010). Furthermore, withdrawal from chronic alcohol exposure increases thresholds for intracranial self-stimulation (Schulteis *et al*, 1995) that are indicative of decreased brain reward function (Koob and Bloom, 1988), and alcohol-dependent animals in acute withdrawal show escalated alcohol consumption that is positively correlated with negative affective-like behaviors, as discussed in the previous work (Williams *et al*, 2012). Collectively, the evidence suggests that negative

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affective states influence alcohol consumption both before and after the development of dependence.

The dynorphin/kappa-opioid receptor (DYN/KOR) system has been implicated in alcohol abuse and dependence, as well as neuropsychiatric disorders including depression (for reviews see Walker *et al* (2012) and Knoll and Carlezon (2010)). Selective KOR antagonists attenuate escalated alcohol self-administration in alcohol-dependent animals (Walker and Koob, 2008), anhedonic- and anxiety-like behavior associated with alcohol and drug withdrawal (Chartoff *et al*, 2012; Valdez and Harshberger, 2012), and anxiety-like phenotypes (Knoll *et al*, 2007). KOR agonists produce conditioned place aversions in rodents (Bals-Kubik *et al*, 1989) and dysphoria in humans (Pfeiffer *et al*, 1986). Furthermore, activation of KORs potentiates the rewarding effect of drugs of abuse, such as nicotine (Smith *et al*, 2012) and cocaine (McLaughlin *et al*, 2006), while mice lacking the prodynorphin gene do not exhibit stress-induced facilitation of cocaine place preferences (McLaughlin *et al*, 2003).

Cues associated with negative affective states that accompany withdrawal produce withdrawal-like symptoms themselves (Kenny *et al*, 2006; Wikler and Pescor, 1967). Thus, negative affective cues may pose as risk factors for the development of AUDs and promote relapse; however, little research has been conducted on the neurobiological basis for this phenomenon. Emerging evidence for the role of the DYN/KOR system in alcohol dependence, fear conditioning (Knoll *et al*, 2007), and relapse to drugs of abuse (Land *et al*, 2009) point to the DYN/KOR system as a possible mediator of cue-induced negative affective-like states.

Previous work has established that measurements of 22-kHz ultrasonic vocalizations (USVs) are an ethologically valid measure of negative affective states (Williams *et al*, 2012; Knapp and Pohorecky, 1995). To further investigate the role of the DYN/KOR system in the production of negative affective states, we evaluated whether the increased 22-kHz USVs observed during acute withdrawal in alcohol-dependent rats could be altered by intracerebroventricular (ICV) infusions of a KOR antagonist. In a complementary experiment, we assessed whether ICV administration of the KOR agonist U50,488 would result in similar increases in the production of USVs as seen in dependent animals. Finally, as evidence suggests that both negative affective states and cues induce escalated consumption of drugs, we assessed whether cues paired with KOR agonism would result in dysregulated alcohol consumption when presented alone and whether such a response was sensitive to KOR antagonism.

MATERIALS AND METHODS

Animals

In total, 90 male wistar rats at least 70 days of age (bred from Charles River Laboratory (Hollister, CA) breeding pairs) were used for all experiments. Animals were group housed (2–3 rats per cage) in a temperature-controlled ($21 \pm 2^\circ\text{C}$) vivarium kept on a 12 h reverse light cycle with *ad libitum* food and water, and were handled for at least 5 days before experimentation. Animal care adhered to the National Research Council's Guide for the Care and Use of Laboratory Animals (1996) and Guidelines for the

Care and Use of Mammals in Neuroscience and Behavioral Research (2003), with all procedures approved by the Washington State University Institutional Animal Care and Use Committee.

Surgeries

All animals were anesthetized with isoflurane gas ($\sim 2\%$) and bilateral guide cannulae were implanted into the lateral ventricles using stereotaxic coordinates (from bregma: DV -3.7 ; AP -0.8 ; and ML ± 1.5) (Paxinos and Watson, 2005). ICV guide cannulae were secured in place using acrylic dental cement and three machine screws were used to anchor the acrylic. The open ends of the guide cannulae were sealed with bilateral obturators (PlasticsOne, Roanoke, VA) to reduce risk of infection and maintain guide cannulae patency. Obturators and cannula guides were protected with stainless-steel cap nuts. Postoperative analgesics (flunixin; MWI Veterinary Supply, Meridian, ID) and antibiotics (Baytril; MWI Veterinary Supply) were administered daily for a total of 5 days following surgery. Animals were allowed to recover for at least 1 week before behavioral testing began. Cannula patency was verified throughout the experiment by ensuring that the proper volume of solution had been infused into each subject during pharmacological testing (with infusions occurring at a rate of $1 \mu\text{l}/\text{min}$) and by histology at the conclusion of the experiment by injecting $1 \mu\text{l}$ 0.6% cresyl violet over 1 min, extracting the brain, and confirming intraventricular dye penetration had occurred.

Intermittent Alcohol Vapor Exposure

Intermittent alcohol vapor exposure (14 h on/10 h off; 7 days/week) induces increased motivational and negative affective states symptomatic of alcohol dependence as evidenced by escalated alcohol self-administration and affective indices such as increased 22-kHz USVs (Nealey *et al*, 2011; Walker and Koob, 2008; Walker *et al*, 2010; Williams *et al*, 2012). The apparatus (La Jolla Alcohol Research, La Jolla, CA) allows blood alcohol levels (BALs) to be titrated by the experimenter by adjusting the rate of 95% alcohol that is vaporized and introduced into the air flow to the sealed environmental chambers in which the animals are housed. This allowed the animal's BALs to be kept within a desired range (175–225 mg%), which was determined semiweekly by collecting blood from the tails ($\sim 50 \mu\text{l}$). After centrifugation, plasma samples were assayed for alcohol content using the Analox AM1 (Analox Instruments, Lunenburg, MA).

Behavioral Measures

Ultrasonic vocalizations. Production of 22-kHz USVs were assessed in a quiet room with dim (15 lx) lighting by administering an air-puff (~ 60 psi) to the nape of the animal's neck. Air-puffs have been validated as a non-painful method of inducing 22-kHz USVs in rodents (Knapp and Pohorecky, 1995). Vocalizations were recorded by a microphone affixed 15 cm above the animal's head. Each test consisted of two trials, and each trial consisted of 15 air-puffs, or until the animal vocalized. Air-puffs were

separated by 15 s and once an animal vocalized, the experimenter waited until the animal stopped vocalizing for 1 min before beginning a new trial. If an animal vocalized for 10 min, the trial was stopped and a new trial began. Vocalizations were recorded with a P48 Electret Ultrasound Microphone (Avisoft Bioacoustics, Germany), E-MU Systems Audio/MIDI Interface (Scotts Valley, CA), and Avisoft Bioacoustics software (created by AEST, Italy). The number and duration of 22-kHz USVs from the second trial were counted with Avisoft Bioacoustics software (Berlin, Germany) and used for all data analyses.

Operant alcohol self-administration. Animals were trained to self-administer a 10% alcohol solution with a sweetener-fade technique (Samson, 1986) during 30 min operant self-administration sessions, as described previously (Nealey *et al*, 2011). Operant training took place in standard operant conditioning chambers (Med Associates, St Albans, VT) with custom drinking wells (Behavioral Pharma, La Jolla, CA) using a continuous schedule of reinforcement. Acquisition of the operant response occurred using a sweetened fluid as the reinforcer (0.125% saccharin and 3% glucose), which is preferred to sucrose (Valenstein *et al*, 1967) and does not require food or water deprivation. A measure of 10% alcohol (w/v) was added to the sweetened fluid, and over 3 weeks, sweetener was slowly removed until only 10% alcohol (w/v) was left in the solution. After responding for alcohol stabilized (<10% deviation in lever-presses over three sessions), the animals underwent ICV cannulation. After recovery from surgery, animals were allowed access to 30 min operant alcohol self-administer sessions that occurred 5 days a week until responding for alcohol re-stabilized.

Experiment 1

In all, 30 alcohol vapor-exposed animals were used to evaluate the effect of the KOR antagonist nor-binaltorphimine (nor-BNI) on air-puff-induced 22-kHz USVs. After ICV cannulation and recovery, animals were randomly assigned to one of four doses of nor-BNI administration (0, 4, 8, or 16 μ g) and placed into alcohol vapor chambers for a 2-week exposure regimen. Following the 2-week vapor exposure duration, animals were infused with nor-BNI or vehicle 24 h before testing that occurred during acute withdrawal (6 h into withdrawal).

Experiment 2

A total of 24 animals were used to evaluate the effect of the KOR agonist U50,488 on air-puff-induced 22-kHz USVs. After ICV cannulation and recovery, animals were assigned to one of four doses of U50,488 (0.0, 0.25, 2.5, and 25 μ g), and 15 min after ICV infusion of U50,488 or vehicle, 22-kHz USVs were measured. Separate groups of animals ($n=7$ /group) were used to assess whether the effect of U50,488 on 22-kHz USVs was sensitive to KOR antagonism by infusing nor-BNI (8 μ g) or vehicle 24 h before U50,488 (25 μ g) pre-treatment that occurred 15 min before 22-kHz USV measurement.

Experiment 3

Five animals were used initially to evaluate whether cues associated with the KOR agonist U50,488 could affect alcohol drinking during a 30-min operant self-administration session. After recovery from surgery, animals were allowed to self-administer alcohol until individual stability was achieved. Before conditioning, almond scent, a neutral stimulus (NS), was presented before self-administration sessions to ensure that the NS alone did not affect alcohol consumption. The almond scent was presented using a gauze pad saturated with 0.5 ml almond extract (McCormick & Company, Hunt Valley, MD) around the upper perimeter of a cage used specifically for conditioning and then leaving the animal in the cage for 15 min. Then, on two consecutive non-self-administration days, an ICV infusion of 25 μ g U50,488 was paired with the almond scent for 15 min. Once the individual animals displayed stable alcohol self-administration rates without the presentation of the cue (up to 12 additional sessions), the almond scent, now considered a conditioned stimulus (CS), was presented alone before a final alcohol self-administration session. Additional groups of animals ($n=5-8$ /group) were used to assess whether KOR antagonism could reverse the cue-induced escalation of operant responding. Operant self-administration and conditioning were conducted as described above. Following the conditioning trials and once stable self-administration was achieved, infusions of artificial cerebrospinal fluid (aCSF) or nor-BNI (1.6 or 16 μ g) occurred 12 h before the final test session. On test day, the CS was presented alone and alcohol self-administration was measured.

Drugs

The KOR agonist U50,488 and the KOR antagonist nor-BNI (both of which have previously been used to modulate the DYN/KOR system; Walker *et al*, 2011; Nealey *et al*, 2011; Land *et al*, 2009) were purchased from Tocris Bioscience (Ellisville, MI) and dissolved in sterile aCSF. Both compounds were prepared daily and infused at a rate of 1 μ l/min ICV.

Statistics

The effects of both nor-BNI in dependent animals and U50,488 in non-dependent animals on number of USVs were analyzed with a one-way univariate analysis of variance (ANOVA). If a main effect of dose was identified, *post-hoc* least significant difference (LSD) tests were conducted. The effect of nor-BNI vs vehicle infusion on U50,488-mediated 22-kHz USV production was analyzed with a univariate ANOVA. To protect against both type I and type II errors, both the significance ($\alpha=0.05$) and power ($1-\beta$, with $\beta=0.2$) were assessed. To avoid the unnecessary use of animals, subjects were added to each experiment in a counterbalanced manner until both appropriate significance and power were achieved.

A univariate ANOVA was conducted on the cue-induced alcohol consumption with a dose of U50,488 or nor-BNI as the between-group factors. All values were expressed as percent change between test day and the previous

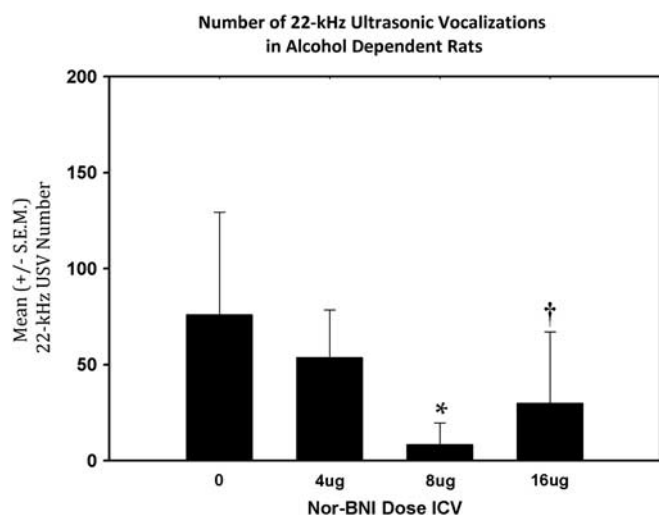


Figure 1 Mean (\pm S.E.M.) number of 22-kHz USVs in dependent animals ($n = 6-9$ /group) after 24 h pre-treatment with nor-binaltorphimine (nor-BNI) (0, 4, 8, or 16 μ g). The two highest doses of nor-BNI decreased the number of USVs (* $p < 0.01$ and † $p < 0.05$).

self-administration session. When a main effect of dose was found, *post-hoc* LSD tests were conducted to compare the experimental treatments to the vehicle condition. If assumptions of normality were not met, nonparametric statistics were employed.

RESULTS

Because of post-surgical complications, four animals were removed from the study. Thus, of the 94 animals that began the study, 90 successfully completed the study and were included in the data analysis.

Figure 1 shows a main effect of nor-BNI dose on the number of 22-kHz USVs emitted in alcohol-dependent animals. The number of USVs decreased in a dose-dependent manner ($F(3,30) = 4.814$, $p < 0.01$) and *post-hoc* LSD tests identified that the 8 and 16 μ g doses of nor-BNI significantly reduced ($p < 0.01$) the number of USVs emitted.

As seen in Figure 2, a main effect of dose ($F(3,23) = 4.446$, $p < 0.05$) was identified for the number of 22-kHz USVs emitted following ICV U50,488 pre-treatment. *Post-hoc* LSD tests identified the 2.5 and 25 μ g dose of U50,488 significantly increased ($p < 0.05$) the number of 22-kHz USVs emitted. Similarly, a main effect of nor-BNI dose ($F(1,12) = 6.530$, $p < 0.05$) was identified (Figure 3) and illustrated that nor-BNI reduced the number of 22-kHz USVs produced by U50,488.

The effect of a cue associated with U50,488 administration can be seen in Figure 4. As the data from this sample was found to be skewed ($Z_{g1} = 2.847$) and leptokurtic ($Z_{g2} = 1.679$), indicating a lack of normal distribution ($p = 9.210$, $\alpha < 0.01$), nonparametric tests were used. The Wilcoxon's signed-rank test found that the percent change between baseline and test day was significantly different from the percent change between baseline and presentation of the NS ($W = 18.00$, $p < 0.05$).

The analysis of affective cue-induced self-administration following ICV KOR antagonism (see Figure 5) revealed a

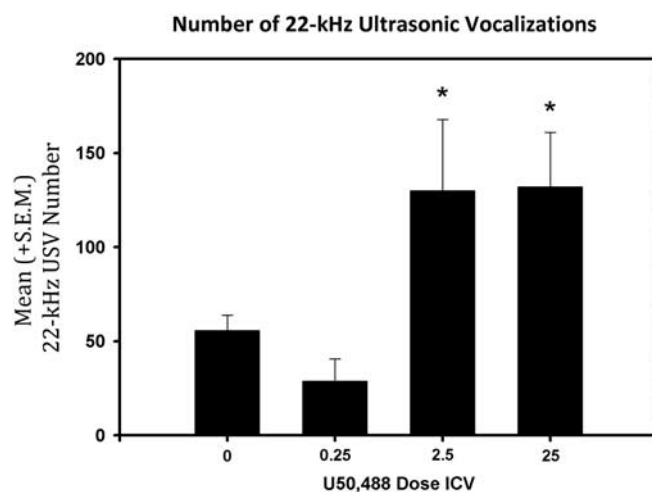


Figure 2 Mean (\pm S.E.M.) number of 22-kHz ($n = 6$ /group) 15 min after intracerebroventricular (ICV) infusion of U50,488 (0, 0.25, 2.5, and 25 μ g). U50,488 dose-dependently increased the number of USVs (* $p < 0.05$).

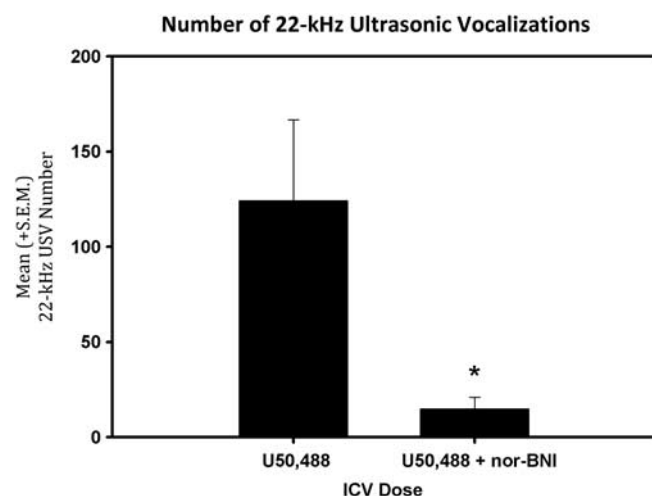


Figure 3 Mean (\pm S.E.M.) number of 22-kHz USVs ($n = 7$ /group) after 24 h pre-treatment with nor-binaltorphimine (nor-BNI) (8 μ g) and 25 μ g of U50,488 15 min before USV measurement. Nor-BNI decreased the number of USVs (* $p < 0.05$).

significant main effect of dose ($F(2,19) = 6.774$, $p < 0.01$). *Post-hoc* LSD tests indicated that the 16 μ g nor-BNI pre-treatment group differed significantly ($p < 0.01$) from the no infusion group as well as the aCSF pre-treatment group. The 1.6 μ g nor-BNI pre-treatment group, however, was not significantly different from the no infusion group or the aCSF pre-treatment group (both $p > 0.05$).

DISCUSSION

Consistent with previous research reporting increased 22-kHz USVs during acute withdrawal in alcohol-dependent rats following 2 weeks of vapor exposure (Williams *et al*, 2012), the alcohol-dependent rats in this study robustly produced 22-kHz USVs during acute withdrawal. For the first time, dependence-induced 22-kHz USVs were shown to be dose-dependently reduced by the KOR antagonist nor-BNI. These data support earlier assertions that the negative

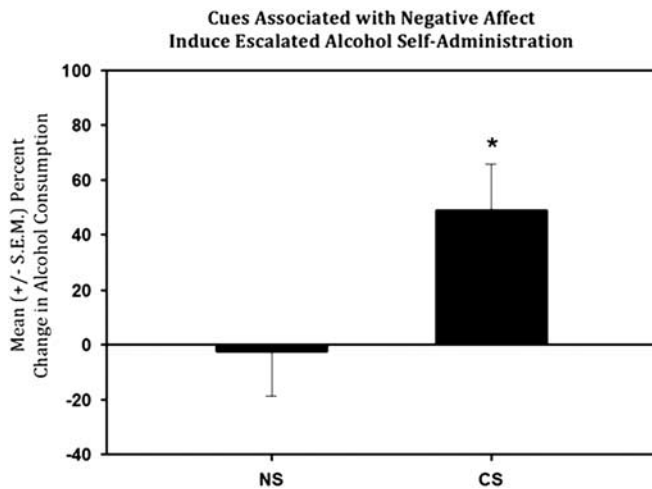


Figure 4 Mean (\pm S.E.M.) percent change in alcohol consumption in non-dependent animals ($n=5$) following presentation of the neutral stimulus (NS), almond scent, or the conditioned stimulus (CS) as compared with baseline responding. Presentation of the CS alone resulted in an increase in alcohol self-administration compared with the stimulus before it being paired with kappa-opioid receptor agonism; * $p<0.05$ when compared with NS.

affect present during withdrawal involves neuroadaptations in the DYN/KOR system that can drive excessive alcohol consumption according to a 'self-medication' hypothesis (Walker *et al*, 2012; Walker and Koob, 2008). Of interest is the previous observation that 2 weeks of vapor exposure seemed to be pro-depressive, rather than anxiety-promoting, and that the increased 22-kHz USVs during withdrawal from 2-week vapor exposure corresponded to a depressive-like phenotype. It follows that since this study used the same 2-week exposure duration, one could posit that the effects of nor-BNI in this study were rescuing the depressive-like phenotype produced during acute withdrawal.

The number of 22-kHz USVs was dose-dependently increased by an ICV infusion of the KOR agonist U50,488, indicating that the KOR agonist can effectively induce a negative affective state in rodents. Specifically, the two highest doses of ICV U50,488 (2.5 and 25 μ g) produced significantly higher numbers of 22-kHz USVs compared with vehicle. Furthermore, the effect of the highest dose of U50,488 (25 μ g) on USVs was significantly attenuated by nor-BNI, affirming that the negative affective state was specific to KORs. Therefore, based on experiments 1 and 2, KOR agonist infusions successfully mimic an alcohol withdrawal-like state, which is reversible by blockade of the KOR.

This is also the first published evidence that a cue paired with a negative affective state can result in the dysregulation of alcohol self-administration. While previously ineffective at altering alcohol consumption, once the NS was paired with the negative affective state produced by U50,488, the CS was able to induce a robust increase in alcohol self-administration. Furthermore, infusion of the 16 μ g dose of nor-BNI before presentation of the CS prevented the ability of the CS to increase alcohol self-administration. The no-infusion group and the aCSF groups did not differ significantly from each other, although they both differed significantly from the highest nor-BNI dose. This indicates that the potentiated drinking produced by affective cues was

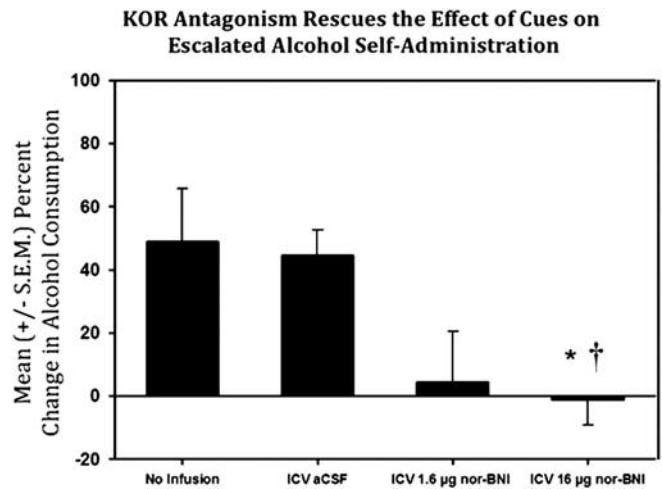


Figure 5 Mean (\pm S.E.M.) percent change in alcohol consumption in non-dependent animals ($n=5-8$ /group) following presentation of the conditioned stimulus (neutral stimulus (NS)), almond scent, as compared with baseline responding in the no infusion group, intracerebroventricular (ICV) artificial cerebrospinal fluid (aCSF) group, 1.6 μ g nor-binaltorphimine (nor-BNI), and 16 μ g nor-BNI. This figure indicates that the escalated self-administration produced by the cue could be blocked by pre-treatment with 16 μ g nor-BNI (a selective kappa-opioid receptor antagonist; * $p<0.05$ when compared with no infusion group and † $p<0.05$ when compared with the aCSF group).

sensitive to nor-BNI, and thus mediated by the DYN/KOR system, this effect was not due to different basal alcohol consumption between the four groups (data not shown).

Other research has also examined the relationship between negative affect and the DYN/KOR system. The KOR antagonist nor-BNI reverses depressive-like phenotypes in the forced swim test (FST) for cocaine withdrawn (Chartoff *et al*, 2012) and has anxiolytic effects in the elevated plus maze (EPM; Knoll *et al*, 2007). Both the EPM and FST are commonly used models of anxiety- and depressive-like behavior, respectively, as they have good predictive validity for anxiolytic and antidepressant drug efficacy (Duman, 2010). Another study showed that while nor-BNI decreases immobility in the FST, the KOR agonist U69 593 increases immobility (Mague *et al*, 2003). U69 593 increases ICSS thresholds in the lateral hypothalamus (Todtenkopf *et al*, 2004), which is indicative of anhedonic behavior, and the KOR agonist U50,488 produces a dose-dependent conditioned place aversions in rats (Bals-Kubik *et al*, 1989; Mucha and Herz, 1985) and increases distress-induced USVs in rat pups (Barr *et al*, 1994) in a nor-BNI reversible manner. Collectively, these data suggest that the DYN/KOR system contributes to negative affective states. These same negative affective states induced by DYN are seen in alcohol withdrawal. It was previously shown that alcohol-dependent rats in withdrawal demonstrate increased prodepressive-like behaviors (Williams *et al*, 2012), such as increased immobility in the FST. In addition, other studies have shown that rats spend increased time in the closed arm of the EPM during alcohol withdrawal (Valdez *et al*, 2004). Therefore, both alcohol withdrawal and KOR agonists increase negative affective states in rodents.

A primary question is whether the DYN/KOR system regulates alcohol withdrawal-induced negative affect. Studies

have supported this link as alcohol withdrawal has been shown to increase DYN levels in both the nucleus accumbens (Acb) and amygdala (Lindholm *et al*, 2000). The same study also found increased DYN levels in the Acb of rats 30 min and 21 days after chronic alcohol exposure, indicating that chronic alcohol consumption leads to both short- and long-term changes in the DYN/KOR system. Interestingly, alcohol-preferring mice (C57BL/6j strain) have lower basal levels of prodynorphin mRNA and KORs in the Acb than alcohol-avoiding mice (Jamensky and Gianoulakis, 1997). Therefore, C57BL/6j mice may prefer alcohol because they experience less of the dysphoric effects produced by the DYN/KOR system following alcohol consumption. In addition, the selective KOR antagonist nor-BNI decreases escalated alcohol self-administration in alcohol-dependent rats (Walker and Koob, 2008; Walker *et al*, 2011; Nealey *et al*, 2011). This evidence suggests that alterations in the DYN/KOR system are important in the physiological and motivational alterations that occur in dependence and withdrawal, including negative affective states that occur in withdrawal (Chartoff *et al*, 2012).

Some evidence indicates that KOR agonists can attenuate place preferences for alcohol (Logrip *et al*, 2009), while other evidence indicates that KOR agonists can potentiate place preferences for alcohol (Sperling *et al*, 2010). This apparent contradiction can be explained based on methodological differences related to the timing of the KOR agonist administration (Walker *et al*, 2012). This study developed its timing parameters for KOR agonist infusion based on those shown to promote increased place preferences (Sperling *et al*, 2010), rather than decreased place preferences.

We hypothesized that cues associated with a DYN-induced negative affective state would increase alcohol consumption in the presence of that cue. Cues associated with negative affective states may pose as risk factors for the initial dysregulation of alcohol consumption and promote relapse. Aside from alcohol dependence and withdrawal, there is emerging evidence for the role of the DYN in fear conditioning, and relapse to drugs of abuse (Land *et al*, 2009). One study showed that nor-BNI could block fear reinstatement, while U50,488 could potentiate fear renewal (Cole *et al*, 2011). Another study found that the KOR antagonists nor-BNI and JDTic could attenuate conditioned fear in the fear-potentiated startle paradigm (Knoll *et al*, 2007). Other research has shown that the KOR agonist CI-977 increased alcohol preference and intake during alcohol deprivation in long-term alcohol-experienced rats (Holter *et al*, 2000), indicating that the DYN/KOR system may be important in relapse to drinking. Furthermore, our data implicate the DYN/KOR system in the initial dysregulation of alcohol self-administration via conditioned affective processes, pointing to activity in the DYN/KOR system serving as a risk factor for developing alcohol dependence.

While the cue associated with KOR activation did result in a significant increase in alcohol self-administration, it should be noted that the cued self-administration levels, when measured in g/kg, resulted in a ~50% increase in alcohol consumption approximating 0.7 g/kg. Based on a previous systematic evaluation of orally administered alcohol and the resulting BALs (Walker and Ehlers, 2009), that level of alcohol consumption would be predicted to produce BALs in the range 70 mg/dl, which is slightly less than the 80 mg/dl threshold previously posited as the ideal

goal for binge and dependence models (Walker *et al*, 2008). However, the current affective cue experiments were not designed to develop a binge-drinking model, but instead to investigate whether affective-linked cues could alter the consumption of alcohol in *non-dependent* animals, which they did. One explanation for this consumption level is that only the k_1 receptor was activated, whereas the pharmacologically validated, but not cloned, k_2 subtype of the KOR (Mansour *et al*, 1994) was not activated by U50,488. Thus, the consumption levels of <80 mg/dl could be due to U50,488 being a selective k_1 ligand, whereas the endogenous DYNs could activate both k_1 and k_2 subtypes of the KOR to induce an increase in negative affect.

DYN's relationship with alcohol and other drugs stems from its ability to modulate other neurotransmitters such as dopamine (DA), GABA, glutamate, and serotonin. Activation of the DYN/KOR system has been found to modify DA in the Acb following cocaine (Thompson *et al*, 2000) and ethanol use (Doyon *et al*, 2006). The DYN/KOR system also modulates dopaminergic neurons projecting to the prefrontal cortex (Margolis *et al*, 2006). The KOR agonist, U69593, leads to a decrease in GABA and glutamate in the Acb (Hjelmstad and Fields, 2003); both neurotransmitters that are important in addiction (Walker and Koob, 2007; Walker and Ettenberg, 2001; Ezequiel and Nobre, 2012). Activation of the p38 mitogen-activated protein kinase system via stress-induced negative affect is KOR dependent (Bruchas *et al*, 2009), and p38 activation also regulates the serotonergic system (Samuvel *et al*, 2005), which has recently been shown to innervate the Acb and is susceptible to KOR regulation (Land *et al*, 2009). Thus, in a single area such as the Acb, KORs have the ability to modulate at least four different neurotransmitters, which identifies a need for research to identify the precise mechanism through which the DYN/KOR system can contribute to dysregulated alcohol consumption.

The results presented here are important because of the high comorbidity of alcoholism with affective disorders such as depression (Boden and Fergusson, 2011), as well as the interaction of negative affect and relapse to alcohol use (Higley *et al*, 2011). Furthermore, understanding how the DYN/KOR system is involved in alcohol withdrawal-induced negative affect and escalated alcohol drinking provides additional targets for pharmacotherapeutic development. In addition to the establishment that withdrawal-induced increases in 22-kHz USVs are sensitive to KOR antagonism and that KOR agonists induce 22-kHz USVs, we are the first to demonstrate that cues associated with the activation of KORs (and the resulting negative affective state) can dysregulate alcohol consumption in non-dependent animals. Importantly, the negative affective cue-induced increases in self-administration were attenuated by KOR antagonism. Collectively, these data indicate that the DYN/KOR system is important in the pathogenesis of alcohol withdrawal-induced negative affect and implicates the DYN/KOR system as a mediator of excessive alcohol consumption related to affective cues in non-dependent rats.

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DISCLOSURE

The authors declare no conflict of interest.

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