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The kappa opioid receptor antagonist JD_{Tic} attenuates alcohol seeking and withdrawal anxiety

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Abstract

The role of kappa-opioid receptors (KOR) in regulation of alcohol-related behaviors is not completely understood. For example, alcohol consumption has been reported to increase following treatment with KOR antagonists in rats, but was decreased in mice with genetic deletion of KOR. Recent studies have further suggested that KOR antagonists may selectively decrease alcohol self-administration in rats following a history of dependence. We assessed the effects of the KOR antagonist JD_{Tic} on alcohol self-administration, reinstatement of alcohol seeking induced by alcohol-associated cues or stress, and acute alcohol withdrawal-induced anxiety (“hangover anxiety”). JD_{Tic} dose-dependently reversed hangover anxiety when given 48 h prior to testing, a time interval corresponding to the previously demonstrated anxiolytic efficacy of this drug. In contrast, JD_{Tic} decreased alcohol self-administration and cue-induced reinstatement of alcohol seeking when administered 2 h prior to testing, but not at longer pretreatment times. For comparison, we determined that the prototypical KOR antagonist nor-BNI can suppress self-administration of alcohol at 2h pretreatment time, mimicking our observations with JD_{Tic}. The effects of JD_{Tic} were behaviorally specific, as it had no effect on stress-induced reinstatement of alcohol seeking, self administration of sucrose, or locomotor activity. Further, we demonstrate that at a 2h pretreatment time JD_{Tic} antagonized the antinociceptive effects of the KOR agonist U50,488H but had no effect on morphine-induced behaviors. Our results provide additional evidence for the involvement of KOR in regulation of alcohol-related behaviors and provide support for KOR antagonists, including JD_{Tic}, to be evaluated as medications for alcoholism.

Keywords

Alcoholism; Dynorphin; Ethanol; Reinstatement; Self-administration; Stress

INTRODUCTION

Recent evidence has indicated a significant role of dynorphin (DYN) and its preferred kappa-opioid receptor (KOR) in stress responses, anxiety, depression, and addiction-related

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Author Contributions

JRS, JAM, JLW, FIC, AT, and MH contributed to conceiving experiments and planning experimental design. JRS, ALG, CEK, and KER performed all alcohol studies and control experiments. JAM and JLW performed analgesia experiments, analyzed data, and composed the related methods and results sections. JRS and MH drafted the initial manuscript and performed all statistical analysis for alcohol studies and control experiments. All authors contributed to reviewing and editing the content of the manuscript.

behaviors (Bruchas et al., 2010; Knoll and Carlezon, 2010). Given the significant overlap between these psychopathologies in clinical populations, this profile suggests the KOR as a potentially useful target for the development of addiction pharmacotherapies.

The role of DYN and KOR in the intersection of stress and addictive behaviors has been studied extensively, but the pattern of results is not entirely consistent. For example, KOR activation is a critical mediator in the ability of repeated stress to enhance drug reward and depressive-like behaviors (McLaughlin et al., 2006; McLaughlin et al., 2003). Furthermore, KOR antagonism can block stress-induced reinstatement of cocaine seeking following extinction (Beardsley et al., 2005; Redila and Chavkin, 2008) and has been shown to suppress cocaine self-administration (Kuzmin et al., 1998). On the other hand, several groups instead report that KOR agonists decrease self-administration of, and conditioned place preference for, various drugs of abuse, including cocaine and heroin (Funada et al., 1993; Glick et al., 1995; Schenk et al., 1999).

Elucidating the role of the DYN/KOR system for alcohol-related behaviors has proven particularly challenging. Following acute administration of high alcohol doses, DYN release was reported in the nucleus accumbens (NAC), and was suggested to mediate aversive properties of alcohol (Marinelli et al., 2006). Seemingly consistent with this observation, KOR antagonists were reported to increase (Mitchell et al., 2005), and KOR agonists to decrease (Lindholm et al., 2001) alcohol consumption in rats. In contrast, however, mice lacking the KOR (Kovacs et al., 2005) or the precursor peptide for DYN (Blednov et al., 2006) consume less alcohol, and KOR agonists increase ethanol consumption under some conditions (Holter et al., 2000). Also, KOR antagonists suppress escalated alcohol self-administration during acute withdrawal in ethanol dependent rats (Nealey et al., 2011; Walker and Koob, 2008; Walker et al., 2011). Adding to the complexity of this relationship, the KOR agonist U50,488H has been found both to block (Logrip et al., 2009) and to potentiate (Sperling et al., 2010) alcohol reward, as measured by conditioned place preference (CPP). Both the level of stress and the history of brain alcohol exposure may be important determinants of the consequences of KOR activation/blockade for alcohol reward and self-administration (Lindholm et al., 2000; Sperling et al., 2010; Walker and Koob, 2008; Nealey et al., 2011; Walker et al., 2011; Holter et al., 2000).

DYN and KOR are widespread in the brain and it is commonly believed that the influence of this system on reinforcement by addictive drugs is mediated, at least in part, by its action in the nucleus accumbens (NAC), where KOR activation provides tonic inhibition of dopamine (DA) release (Shippenberg et al., 2007; Spanagel et al., 1992). However, it is important to consider that the KOR is highly expressed in multiple regions of the amygdala (Le Merrer et al., 2009), co-localizes with corticotropin-releasing hormone [CRH; (Marchant et al., 2007)], and appears to serve as a downstream link with CRH in conditioned aversive responses (Land et al., 2008). KORs in the dorsal raphe nucleus have also been shown to play a functional role in the expression of aversive behaviors (Land et al., 2009). The presence of DYN/KOR in these structures, and their differential contribution to behavioral output under varying conditions, may account for the complex consequences of KOR blockade.

To date, a number of KOR antagonists have been introduced with varying degrees of specificity and duration of action (see Melief et al., 2011), such as zyklophin (Aldrich et al., 2009), arodyn (Bennett et al., 2002), or nor-binaltorphimine [nor-BNI; (Portoghese et al., 1987)]. Recently, JDTic was developed to provide specific, long lasting inhibition of the KOR (Carroll et al., 2004). Initial assessments of JDTic activity indicated that this compound has an increased potency compared to previously developed KOR antagonists, and has excellent selectivity for the KOR, especially when compared to its action at the

DOR (Thomas et al., 2003; Thomas et al., 2001). Most reports thus far suggest that JD_{Tic} has a slow onset of action (6 hours), and a long lasting inhibitory activity, ranging up to 4 weeks (Carroll et al., 2004), that may be mediated by actions on c-Jun N-terminal kinases [JNKs; (Bruchas et al., 2007; Melief et al., 2011; Melief et al., 2010)]. JD_{Tic} has been shown to possess anxiolytic and antidepressant-like activity in rodent models, and is capable of blocking the expression of stress-induced reinstatement of cocaine seeking (Knoll et al., 2007; Beardsley et al., 2005). The current studies were designed to assess the effects of JD_{Tic} on alcohol self-administration, alcohol seeking, and alcohol withdrawal anxiety. For comparison, we also assessed the effect of the prototypical KOR antagonist nor-BNI on alcohol self-administration.

METHODS

Animals

Male Wistar rats weighing approximately 175 to 225 g at time of arrival were obtained from Charles River (Wilmington, MA). Rats were allowed at least one week to habituate to the housing facility and were handled daily for a week prior to the start of experimentation. For all studies except for antinociceptive testing, rats were housed in a reversed light cycle (lights on 20:30, lights off 08:30), and all testing took place during the dark phase. For antinociceptive testing, rats were housed in a 12 h light:dark cycle and were tested during the light phase. Food and water were available *ad libitum*, except where explicitly stated. All procedures used were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the NIH or RTI Animal Care and Use Committee.

Hangover Anxiety

The elevated plus maze is a commonly used behavioral paradigm to assess anxiety-like behavior. The apparatus used in these experiments consisted of 4 arms of 20 in length and 4 in width. The arms were configured in a plus orientation and were elevated 28 in above the floor. Two arms had a 16 in wall enclosing the arm, while the other two arms were open with a ½ in lip around the platform of the arm. Alcohol naïve rats (n=11–12 per group) were injected with a bolus dose of alcohol (3 g/kg, i.p., 20 % alcohol, v/v, in saline), and tested on the elevated plus-maze 12 h later as described previously (Gehlert et al., 2007). JD_{Tic} (0, 3, or 10 mg/kg, i.p. at 1 ml/kg, dissolved in sterile water) was administered 48 h prior to plus-maze testing (36 h prior to alcohol injection). Plus maze tests were conducted under red light and were 5 minutes in duration. Behavioral activity was recorded and analyzed using the Noldus Ethovision system. The pretreatment interval and doses were selected based on previous reports of JD_{Tic} anxiolytic effects (Knoll et al., 2007).

Alcohol Self-Administration Training and Testing

Standard operant chambers (Med Associates, St. Albans, VT) were used in these experiments. Chambers consisted of a wirebar floor enclosed within a plexiglass chamber with two levers on one wall and a receptacle for delivery of solutions located between the two levers. The chamber was situated inside of a larger, sound attenuating box equipped with a fan to circulate air into the apparatus. Self-administration training and testing were as described [e.g. (Cippitelli et al., 2010)]. In brief, a saccharin fading procedure was used, and animals were trained to acquire stable rates of lever pressing for 10 % alcohol (v/v) in water. Training began with 0.2% saccharin solution and the concentration of alcohol in this solution was gradually increased, with intervening sessions during which alcohol was not diluted in saccharin, until a final concentration of 10% ethanol in water was reliably self-administered. Animals were water restricted for 22 hours prior to self-administration sessions for the first 3 days, but then had access to water *ad libitum* for the rest of the experiment. Alcohol solution was delivered into a drinking receptacle in a 0.1 ml volume, on

a fixed ratio 1 (FR1) schedule, during 30 min sessions that were run 5–6 days/week. Each alcohol delivery was followed by a 5 s timeout interval during which a house light was illuminated and responses were recorded but not reinforced. Under these conditions, we typically observe self-administration rates that lead to consumption of approximately 0.8 g/kg of alcohol over the 30 minute session. Reinforcement schedules were controlled, and behavioral responses were recorded, by MED-PC IV software (Med Associates, St. Albans, VT). (For cue-induced reinstatement experiments, orange scent was also present in the self-administration chambers during alcohol sessions.) After stable response rates were reached for at least 3 consecutive days (defined as no more than 15% variation from the mean over the last 3 sessions, which was achieved after approximately 3 weeks), rats in self-administration experiments were injected with JDTC (0, 3, or 10 mg/kg; i.p. at 1 ml/kg, dissolved in sterile water, n=11 per group) or nor-BNI (0 or 30 mg/kg, s.c. at 3 ml/kg, dissolved in sterile saline, n=14 per group) 2 h prior to the next self-administration session. Sessions were continued without additional treatment for 2 more days to provide 24 and 48 h post-injection time points. Each rat received only one treatment dose, in a mixed design with drug dose as a between subjects, and time after treatment as a within subjects factor.

Extinction & Reinstatement

Reinstatement experiments were carried out as described (Cippitelli et al., 2010). After 14–16 days of self-administration, responding was extinguished over a minimum of 15 sessions. Animals that fulfilled an extinction criterion of less than 20 active lever responses in the 30 min session were used for reinstatement testing. Animals that had not at that point reached the criterion received additional extinction sessions, up to a total of 19. Animals that did not reach the criterion at session 19 were discarded (2 out of 42 in the stress induced reinstatement and 2 out of 45 in the cue-induced reinstatement experiment). For cue-induced reinstatement, the light cue and orange scent were removed during the extinction phase.

In the cue-induced reinstatement experiment (n=14–15 per group), presentation of orange scent and the activation of the house light following active lever press served as the reinstatement stimulus. In the stress-induced reinstatement experiment (n=13–14 per group), 10 min of intermittent footshock (0.5 s shock, 0.4 mA intensity, mean intershock interval of 40 s) was delivered in the operant chamber immediately before the reinstatement session. The duration and intensity of shock were lower than commonly used parameters [0.6 mA, 15 min; see e.g. (Le et al., 1998; Shaham and Stewart, 1995)], based on pilot experiments (see Results section). Rats were injected with JDTC (10 mg/kg, i.p. at 0.5 ml/kg, dissolved in sterile water) or vehicle at either 2 or 24 h prior to reinstatement testing. These pretreatment times were selected based on previously published findings reporting effects of JDTC on stress-induced reinstatement of cocaine seeking (Beardsley et al., 2005). Each rat received only one injection, with drug treatment (vehicle, 2 h JDTC, or 24 h JDTC) as a between subjects factor. For both cue- and stress-induced reinstatement, 2 h vehicle and 24 h vehicle groups were generated, in order to match the pre-treatment intervals of the active treatment groups. However, when these vehicle groups were compared, no differences were found. Data for these two groups were therefore combined into a single “vehicle” control group for each experiment.

Sucrose Self-Administration

Rats were water restricted for 22 h for the first 3 days of training, as in self-administration training described above, and were allowed access to 10 % sucrose solution (w/v, dissolved in tap water) on an FR1 reinforcement schedule during initial self-administration sessions. After achieving consistent response rates (after 4–5 days of training on the FR1 schedule), rats were kept on the FR1 schedule, but a 20 s timeout was imposed following sucrose delivery. These conditions were in place for 10 to 13 days, after which time baseline

responding for each group reached a stable level, which was defined as at least 3 consecutive days of no more than 15% variation from the mean over the last 3 sessions. All self-administration sessions were 30 min in duration and were run once daily, 5 – 6 days/week. Rats were then injected with JD_{Tic} (0 or 10 mg/kg, n=7–8 per group) or nor-BNI (0 or 30 mg/kg, n=8 per group), 2 h prior to the next self-administration session. Sessions were continued for 2 more days to provide 24 and 48 h post-injection time points. Each rat received only one injection in a mixed design with post-injection interval as a within-subjects, and drug treatment as a between-subjects factor.

Tail Flick Nociception

Rats (n=6–12/group) were tested on a standard tail flick apparatus (Stoelting Co., Wood Dale, Illinois). After a 6 day habituation period, rats were randomly assigned to one of six conditions [Vehicle/Vehicle; JD_{Tic} (10 mg/kg)/Vehicle; Vehicle/U-50,488H (20 mg/kg); Vehicle/Morphine (10 mg/kg); JD_{Tic} (10 mg/kg)/U-50,488H (20 mg/kg); JD_{Tic} (10 mg/kg)/Morphine (10 mg/kg)]. On test day, each rat was evaluated for baseline latency to remove its tail in the tail flick apparatus prior to any injections. The maximum possible latency was set to 10 s; therefore, if the rat did not flick its tail within 10 s, the heat automatically turned off. Rats were then injected with vehicle or JD_{Tic}, depending on group assignment (see above). Two hours later, rats were injected with vehicle, U-50,488 or morphine, again depending on group assignment. Rats were then tested in the tail flick procedure again 30 min later (for groups that received morphine as a second injection) or 15 min later (for groups that received U50,488H as a second injection). For animals that received saline as a second injection, testing took place 15 or 30 minutes later. The results for 15 and 30 minute pretreatment were compared for each treatment group and did not differ statistically. Therefore, they were combined to form a single saline group. Antinociception was calculated as percent of maximum possible effect (% maximal possible effect = [(test–control latency)/(10–control)] x 100).

Locomotor Activity

This experiment was conducted in standard locomotor activity testing chambers (Med Associates, St. Albans, VT). The arena used for testing measured 17 ½ in by 17 ½ in, and was enclosed by a plexiglass wall measuring 12 in in height. Infrared sensors were located 1 in apart and spanned the perimeter of the testing arena. The arena was enclosed in a larger, sound attenuating box equipped with a fan to circulate air into the testing chamber. Testing took place during the dark phase and chambers were darkened during behavioral measurement. Rats (n=6 per group) were injected, in a between-subjects design, with JD_{Tic} (0, 3, or 10 mg/kg, i.p. at 1 ml/kg, dissolved in sterile water), 2 h prior to the start of the locomotor activity session, which lasted for 1 h. Behavior was measured using infrared beam breaks and was recorded by MED-PC software. Data are expressed as total session activity, as well as 6 consecutive 10 min bins.

Morphine-induced Locomotion

Rats were placed in the test chambers and then injected with morphine (1 mg/kg, s.c. at 1 ml/kg, dissolved in saline, n=9 per group) 30 minutes later. Preliminary experiments in our laboratory under these conditions indicated that the dose of morphine selected fell on the ascending limb of the dose-response curve for morphine-induced locomotion. Locomotor activity was measured for 60 minutes following morphine injection according to the procedures outlined above. JD_{Tic} or vehicle (sterile water) was administered 2 hours prior to morphine injection.

Drugs

JD_{Tic} (Research Triangle Institute, Research Triangle Park NC) was dissolved in sterile water and injected i.p. at a volume of 0.5 or 1.0 ml/kg. nor-BNI (RTI) was dissolved in sterile physiological saline and injected s.c. at a volume of 3 ml/kg. The KOR agonist U50,488H and the MOR agonist morphine were obtained via the NIDA Drug Supply Program.

Statistics

Data were analyzed using ANOVA models specified in details for each analysis in the Results section. Data were evaluated for homogeneity of variance, and when significant departures from this assumption were detected, results were re-analyzed using non-parametric tests as specified in Results, to examine the robustness of findings.

RESULTS

JD_{Tic} reverses acute alcohol withdrawal-induced anxiety

One way ANOVA with treatment as a between subjects factor showed a significant treatment effect ($F(3,42)=4.0$, $p=0.01$, $n=11-12$ /group; figure 1). Post-hoc analysis using Dunnett's test showed that both the Vehicle - Alcohol ($p=0.010$) and JD_{Tic} 3 mg/kg - Alcohol ($p=0.045$) groups differed significantly from the Vehicle - Vehicle group, while the JD_{Tic} 10 mg/kg -Alcohol group did not ($p=0.72$). Specifically, alcohol administration induced an increase in anxiety-like behavior that was returned to control levels by pretreatment with 10 mg/kg, but not 3 mg/kg JD_{Tic}. As a measure of general locomotor activity, total entries were also compared between groups, and no differences were found (data not shown). Thus, JD_{Tic} is capable of reversing anxiety-like behavior in our model of hangover anxiety at a 48 h pretreatment time, in agreement with the previously described anxiolytic efficacy of this compound as measured by the elevated plus-maze (Knoll et al., 2007).

JD_{Tic} decreases alcohol self-administration

Two way ANOVA with post-injection interval as a within subject factor and drug treatment as a between subjects factor showed a significant main effect of post-injection interval ($F(3,90)=6.25$, $p<0.001$, $n=11$ /group; figure 2), and a post-injection interval x treatment interaction ($F(6,90)=2.6$, $p=0.02$). Post-hoc analysis using Newman-Keuls test indicated a significant difference from baseline responding only in the JD_{Tic} 10 mg/kg pretreated group at the 2 h pretreatment time point ($p=0.002$). Furthermore, there was also a significant decrease observed in the 10 mg/kg JD_{Tic} treated group when compared to vehicle control at the 2 h time point ($p=0.039$), while the groups were virtually identical at later time points. To assess the possibility of rate-dependent effects, we have analyzed whether there is a correlation between baseline response rates and the percent decrease on the first treatment day in 10 mg/kg JD_{Tic} treated rats. There was no correlation between these two measures ($r^2=0.27$, $p=0.10$), indicating that rate-dependent effects are unlikely. When considering g/kg concentration consumed during the 30 minute session, identical effects of JD_{Tic} were found. Specifically, two-way ANOVA revealed a main effect of post-injection interval ($F(3,90)=6.00$, $p<0.001$) and a post-injection interval x treatment interaction ($F(6,90)=2.66$, $p=0.02$). Post-hoc Newman-Keuls test indicated a significant difference from baseline responding only in the JD_{Tic} 10 mg/kg pretreated group at the 2 h time point (0.003). There was also a significant decrease observed in the 10 mg/kg JD_{Tic} pretreated group when compared to vehicle control at the 2 h time point (0.02). These results indicate that JD_{Tic} is capable of decreasing alcohol self-administration in rats when given 2 h prior to testing, but not at longer pretreatment times.

Nor-BNI decreases alcohol self-administration

To further explore the role of the KOR in the alcohol self-administration paradigm, we assessed the effect of the commonly used KOR antagonist, nor-BNI. Initial experiments using a commonly used dose of nor-BNI, 10 mg/kg, produced a slight, but not statistically significant suppression of alcohol self-administration rates (data not shown). Given the suggestive results of this experiment, and considering that Knoll and colleagues (2007) saw no off target behavioral suppression with doses of nor-BNI up to 30 mg/kg, we evaluated the effect of this higher dose of nor-BNI. Two way ANOVA with post-injection interval as a within subject factor and drug treatment as a between subjects factor showed a significant main effect of treatment ($F(1,26)=4.4$, $p=0.04$, $n=14/\text{group}$; figure 3), and a post-injection interval $\times$ treatment interaction ($F(3,78)=4.9$, $p=0.004$). Post-hoc analysis using Newman-Keuls test indicated a significant difference from baseline responding only in the nor-BNI 30 mg/kg pretreated group at the 2 h pretreatment time point ($p=0.009$). Furthermore, there was also a significant decrease observed in the 30 mg/kg nor-BNI treated group when compared to vehicle control at the 2 h time point ($p=0.003$), while the groups were virtually identical at later time points. Identical results were found using g/kg consumption as the dependent variable. Specifically, two-way ANOVA revealed a main effect of drug treatment ($F(1,26)=7.89$, $p=0.009$) and a post-injection interval $\times$ treatment interaction ($F(3,78)=4.56$, $p=0.005$). Newman-Keuls post-hoc tests indicated a significant difference from baseline responding only in the nor-BNI pretreated group at the 2 h time point ($p=0.006$). There was also a significant decrease observed in the nor-BNI treated group when compared to the vehicle treated group at the 2 h time point ($p<0.001$). These results indicate a significant suppression of alcohol self-administration following pretreatment with nor-BNI that was present exclusively at the 2 h pretreatment time, paralleling the effect observed with JD_{Tic}.

Neither JD_{Tic} nor nor-BNI affect self-administration of 10 % sucrose

Two way ANOVA with post-injection interval as a within subject factor and JD_{Tic} treatment as a between subjects factor showed significant main effect of post-injection interval ($F(3,39)=5.1$, $p=0.005$; figure 4), likely resulting from slight variations in self-administration rates over the days of measurement, combined with low group variability. There was, however, no main effect of treatment ($F(1,13)=0.02$, $p=0.9$), nor a post-injection interval $\times$ treatment interaction ($F(3,39)=0.175$, $p=0.91$). Accordingly, post hoc analysis did not show significant differences between treatment groups on any session; in fact, treatment groups were virtually identical within each respective session. These results indicate that JD_{Tic} did not have non-specific effects on performance or motivation for a natural reward that may confound results of experiments measuring drug reinforcement using operant behavioral testing.

Experiments evaluating the effect of nor-BNI on sucrose self-administration produced similar results. Two way ANOVA with post-injection interval as a within subject factor and nor-BNI treatment as a between subjects factor showed no significant main effect of post-injection interval ($F(3,42)=1.10$, $p=0.40$; figure 5) or treatment ($F(1,14)=1.20$, $p=0.29$), nor a post-injection interval $\times$ treatment interaction ($F(3,42)=2.60$, $p=0.07$). Accordingly, Newman-Keuls post hoc analysis did not show significant differences between treatment groups on any session, nor any change in responding when comparing nor-BNI treatment at any pretreatment interval to baseline responding ($p>0.6$ for all session comparisons in nor-BNI treated group and $p>0.25$ for all comparisons between treatments for each session). These results indicate that nor-BNI at 30 mg/kg did not have non-specific effects on performance or motivation for a natural reward that could have confounded results of experiments measuring drug reinforcement using operant behavioral testing.

JDTic suppresses cue-induced reinstatement of alcohol seeking

The presence of a reinstatement effect was first determined, shown as a significantly higher response rate following exposure to cues that were previously paired with alcohol availability compared to extinction responding in the vehicle-treated group (repeated measures ANOVA: $F(1,14)=37.04$, $p<0.0001$; figure 6). Because variances were not homogenous between extinction and reinstatement responding, this analysis was repeated using Wilcoxon paired signed-rank test, but the results were essentially identical (not shown). Inactive lever responding was not significantly affected by alcohol paired cues, although there was a trend for an increase ($F(1,14)=3.37$, $p=0.09$), presumably reflecting previously described response generalization (Shalev et al., 2002).

Although groups were matched for extinction responding rates, these remained a significant covariate in the analysis of reinstatement responding on the alcohol-associated lever, and their inclusion reduced the residual variance. Therefore, extinction responding was retained as a covariate in the final model. One way ANOVA with treatment as a between subjects factor showed a main effect of treatment on responses on the alcohol associated lever ($F(2,39)=3.8$, $p=0.03$). Post hoc Newman-Keuls analysis showed that 2 h pretreatment with JDTic (10 mg/kg) significantly decreased reinstatement responding compared to both the vehicle treated group ($p=0.03$) and the group treated with JDTic 24 h prior to reinstatement testing ($p=0.035$). There was no effect of JDTic treatment on inactive lever responding ($F(2,39)=1.12$, $p=0.34$).

JDTic does not affect stress-induced reinstatement

This experiment was initially carried out, in two replications (combined $n=26-28$ /group), that used standard parameters in our laboratory of 15 min of intermittent footshock at 0.6 mA. In these experiments, JDTic (10 mg/kg) did not suppress footshock-induced reinstatement. In fact, under these conditions, reinstatement responding appeared to be slightly increased by JDTic (data not shown). To address the possibility that JDTic might be facilitating lever pressing by attenuation of freezing induced by footshock (i.e., an anxiolytic effect), we repeated the experiment with a milder footshock stressor, 10 min of intermittent footshock at 0.4 mA ($n=13-14$).

In the final experiment, the presence of a reinstatement effect was first detected as a significantly higher response rate following exposure to the intermittent footshock compared to the non-shock condition in the vehicle-treated group (repeated measures ANOVA: $F(1,12)=14.4$, $p<0.01$; similar result on Wilcoxon paired signed-rank test; figure 7). Inactive lever responding was not significantly affected by intermittent footshock stress ($F(1,12)=2.85$, $p=0.12$).

In these experiments, extinction responding was not a significant co-variate in the analysis of reinstatement responding on the alcohol-associated lever, nor did inclusion of this variable reduce the residual variance; extinction responding was therefore dropped from the final model. One way ANOVA with treatment as a between subjects factor did not show any effect of treatment on alcohol associated lever responding ($F(2,37)=0.48$, $p=0.62$). Responses on the inactive lever were also unaffected by drug treatment ($F(2,37)=0.43$, $p=0.65$). Taken together, these results suggest that JDTic, at a dose that has potent-anxiolytic like activity under some conditions, has no effect on stress-induced reinstatement of alcohol seeking in our model.

JDTic specifically blocks the effects of a KOR agonist at the 2 h pretreatment time point

One way ANOVA with treatment as between subjects factor showed a robust treatment effect [$F(5,54) = 51.89$; $p < 0.0001$; figure 8] on tail flick nociception. Dunnett's post-hoc

test showed that the Saline/U50,488H (20 mg/kg) and Saline/Morphine (10 mg/kg) groups were both significantly different from the Saline/Saline group ($p < 0.05$), indicating that both morphine and U50,488H produced analgesia as measured by the tail flick nociception test. The JDTic (10 mg/kg)/Morphine (10 mg/kg) group also differed significantly from the Saline/Saline group ($p < 0.05$), but not from the Vehicle/Morphine group, indicating that JDTic did not prevent μ -opioid receptor (MOR) mediated analgesia. In contrast, the JDTic/Saline and JDTic/U50,488H groups were not significantly different from the Saline/Saline group, showing that JDTic by itself had no analgesic properties and that JDTic blocked KOR-mediated analgesia, respectively. Taken together, these results demonstrate that JDTic blocked KORs at a short pretreatment time of 2 h, and that the inhibitory effects of JDTic at this timepoint are specific for KORs and do not extend to any action at the MOR.

JDTic does not affect locomotor activity or morphine-induced locomotor activation

The results of the JDTic experiment were analyzed in two separate analyses. On a one way ANOVA with treatment as between subjects factor, JDTic did not affect total locomotor activity over one hour as measured by distance traveled ($F(2,17)=0.134$, $p=0.88$, Figure 9A). Data were then analyzed in 10 min bins, with treatment as a between subjects factor, and time as a within subjects factor (Figure 9B). On this analysis, there was a main effect of bin ($F(5,75)=102.5$, $p < 0.0001$), showing that rats introduced to the novel environment showed a high initial level of exploratory activity that gradually decreased over time as the animals habituated to the testing chambers. There was, however no main treatment effect ($F(2,75)=0.13$, $p=0.88$), nor a bin x treatment interaction ($F(10,75)=0.3812$, $p=0.95$). These results indicate that JDTic did not have any non-specific sedative effects that could account for suppression of lever pressing, and also that it did not influence exploratory activity in a novel environment.

JDTic, when administered 2 h prior to morphine injection, had no effect on morphine-induced locomotor activation. One way ANOVA revealed a significant main effect of treatment ($F(2,26)=6.3$, $p=0.006$, $n=9$ per group, figure 10). Post-hoc Newman-Keuls tests indicated that locomotion was elevated in both the vehicle/morphine ($p=0.01$) and JDTic/morphine ($p=0.008$) groups when compared to vehicle/vehicle treated rats. There was no significant difference between locomotion in the vehicle/morphine and JDTic/morphine groups. These findings are important because in the tail flick analgesia experiment, morphine effects were maximal, so it is possible that a partial inhibition of MOR signaling by JDTic would not be detected. Further, morphine antinociception in the tail flick procedure likely acts via spinal MORs, while locomotor activity is regulated by supraspinal circuitry. The lack of JDTic effect on a non-maximal dose of morphine in locomotor activity experiments further supports our interpretation that JDTic at 2 h after administration lacks effect on MOR signaling. This agrees with the findings of Jackson and colleagues (Jackson et al., 2010), who observed no effect of JDTic 1 h after injection on a non-maximal morphine-induced analgesia.

DISCUSSION

We report here that the KOR antagonist JDTic reverses alcohol withdrawal anxiety, suppresses alcohol self-administration, and blocks cue-induced reinstatement of alcohol seeking following extinction. The effects of JDTic on self-administration and cue-induced reinstatement were observed 2 h following injection, a shorter pretreatment interval than previously reported for other JDTic effects. To our knowledge, this is the first assessment of the effect of KOR antagonists on reinstatement of alcohol seeking. At the 2 h time point, JDTic had no effect on sucrose self-administration or locomotor activity, indicating that its effect to decrease alcohol self-administration was behaviorally specific. Because an early onset of JDTic activity in the alcohol self-administration model may be considered

unexpected in view of prior findings (see below), we assessed whether the relatively high self-administration rates in our experimental subjects may have contributed to an increased sensitivity to drug treatment. To assess this possible source of confound, we examined whether individual baseline, pre-treatment response rates correlated with the magnitude of suppression induced by JDTC. We did not find any such correlation, arguing against a rate-dependent activity of the drug. Finally, off-target activity of JDTC at MORs could conceivably account for suppression of both alcohol self-administration (Samson and Doyle, 1985) and cue-induced reinstatement of alcohol-seeking (Liu and Weiss, 2002), we confirmed that JDTC blocked KORs at 2 h, but had no effect on morphine activation of MOR at this time point. JDTC also reversed alcohol-withdrawal anxiety after a longer pretreatment time, 48 h, which is in agreement with previous findings (Knoll et al., 2007). Together, our observations indicate that JDTC can suppress alcohol self-administration as well as relapse-like behavior triggered by alcohol-associated cues, and that these effects of JDTC have an earlier onset than previously reported for other behavioral measures.

Early onset of JDTC activity at 2 h in these experiments is seemingly at odds with prior findings in mice, where JDTC did not reverse KOR mediated analgesia until 6 h after injection (Carroll et al., 2004). However, in an assessment of KOR agonist-induced polyuria in rats, JDTC did show antagonist activity within the first 5 h of treatment (Beardsley et al., 2005; Carroll et al., 2004). Also, JDTC prevented the analgesic effects of the KOR agonists U50,488H (rats, 2 h JDTC pretreatment) and salvinorin A (mice, 1 h JDTC pretreatment) at short pretreatment times [present study; (Walentiny et al., 2010)]. The onset of JDTC activity in rodents may therefore be faster than has been anticipated, and not dissimilar to that of the recently developed KOR antagonist zyklophyn (Aldrich et al., 2009). Early onset of JDTC activity in some behavioral models may not have been detected previously simply because short pretreatment intervals were not included, based on the widespread notion that KOR antagonists mostly have slow-onset, long lasting effects.

Perhaps more unexpected than the early onset is the short duration of action observed both in the self-administration and the cue-induced reinstatement experiments, contrasting with the observations of much longer lasting anxiolytic-like JDTC effects in previous work (Knoll et al., 2007), and in our own hangover-anxiety experiment. This dissociation suggests that the pathways that mediate the anxiolytic-like effects of JDTC and its actions to suppress alcohol self-administration are distinct from one another. Effects of KOR antagonists on acute drug reinforcement may be related to their ability to acutely increase DA release in NAC by relieving tonic dynorphin inhibition (Spanagel et al., 1992), an ability that interacts with a history of repeated alcohol intake (Lindholm et al., 2007). In contrast, the long duration of anxiolytic-like activity may be related to an ability of KOR antagonists to produce long-lasting effects mediated through phosphorylation of JNK kinases (Bruchas et al., 2007), presumably occurring within other brain structures, such as the amygdala or the dorsal raphe. These long-term effects may not occur within the NAC, because in this structure, KORs are found as presynaptic heteroreceptors on DA terminals that also carry D2 autoreceptors (Shippenberg et al., 2007), and increased D2 activation could counteract long-term JNK-mediated effects (Chen et al., 2008). Alternatively, it is possible that long term regulation of JNK phosphorylation impacts some behaviors (such as anxiety and analgesia) but not others (such as primary reinforcement and cue elicited reinstatement). Other studies have observed a similar discrepancy in the duration of KOR antagonist effects. Using measures of alcohol consumption or self-administration, other groups have observed a shorter duration of nor-BNI effect than has been observed in measures of KOR mediated analgesia (Logrip et al., 2008; Walker et al., 2011; Williams and Woods, 1998). This suggests that the duration of activity of KOR antagonists may differ depending on the behavioral measure employed and/or the functional state of the DYN/KOR system.

Previous studies suggest a complex role for KORs in regulation of alcohol intake, self-administration and reward. Using two-bottle free-choice drinking procedures in rats, it was reported that the prototypical KOR antagonist nor-BNI increased alcohol consumption (Mitchell et al. 2005), while the KOR agonist U50,488H suppressed it (Lindholm et al., 2001). In contrast, mice lacking KOR or DYN were found to consume less alcohol than wild-type animals (Blednov et al., 2006; Kovacs et al., 2005), although the latter finding was not replicated in a subsequent study (Sperling et al., 2010). Similarly, one recent study reported that KOR activation blocked both acquisition and expression of alcohol CPP (Logrip et al., 2009), while another instead found marked potentiation of alcohol CPP by the KOR agonist U50,488H. The latter also found evidence for a potentiation of alcohol reward upon exposure to forced swim stress, and established that this effect is likely to be mediated by the endogenous KOR ligand DYN, since it was prevented by nor-BNI (Sperling et al., 2010). Together, these data indicate that effects of KOR antagonists on alcohol-related behaviors may be determined by the functional state of the DYN/KOR system. Specifically, under conditions when this system is activated by stress or other stimuli, KOR antagonists might be particularly potent in blocking alcohol reward and self-administration. An activation similar to that induced by stress exposure may occur during alcohol withdrawal (Lindholm et al., 2000), and contribute to rendering dependent animals particularly sensitive to suppression of self-administration by nor-BNI, as recently reported (Walker and Koob, 2008).

However, results that we present here suggest that the KOR also contributes to regulation of baseline alcohol reinforcement. We observed a suppression of responding for alcohol following pretreatment with both nor-BNI and JDTC. This is seemingly at odds with previous findings from Walker and Koob, who found no effect of nor-BNI in non-dependent rats (Nealey et al., 2011; Walker and Koob, 2008; Walker et al., 2011). However, there were several differences in the specific protocols used in their experiments when compared to our studies (lower nor-BNI dose when given systemically, choice between ethanol and water during self-administration sessions, lower response rates in non-dependent rats). We observed a significant suppression of self-administration in our experiments using 30 mg/kg nor-BNI, a dose which has been given by other groups previously with no non-specific effects (Knoll et al., 2007). We also confirm with sucrose self-administration experiments that this dose of nor-BNI does not induce a suppression of responding for a natural reinforcer. Interestingly, studies using both mice and non-human primates have demonstrated an inhibition of alcohol self-administration at short post treatment intervals following nor-BNI injection, similar to our observations (Williams and Woods, 1998; Logrip et al., 2008). At any rate, our present findings suggest that JDTC can suppress alcohol self-administration in the absence of stress-or withdrawal induced activation of the DYN/KOR system, with a short duration effect. We would, however, make the prediction that JDTC would be more effective at suppressing alcohol self-administration and reinstatement of alcohol seeking in alcohol dependent animals (see discussion above).

Unexpectedly, JDTC blocked reinstatement of alcohol-seeking triggered by alcohol associated cues. This was also a rapid onset effect. In the stress- and cue-induced reinstatement experiments, we initially planned to include only a 24 h pretreatment time, as used in a prior reinstatement study with JDTC (Beardsley et al., 2005). In view of the results of the self-administration experiment, however, it became apparent that a 2 h pretreatment time must also be evaluated in the reinstatement studies. As with self-administration, we observed a suppression of cue-induced reinstatement when JDTC was given 2h, but not 24 h, prior to testing. To our knowledge, this is the first finding that KOR antagonism suppresses cue-induced reinstatement of drug seeking for any class of drug. As in the case of the self-administration finding, our observations of unaffected lever-pressing for sucrose with the same doses and the same pre-treatment interval, as well as the lack of effects on

stress-induced reinstatement, provide stringent controls for the possibility that the effects of JD_{Tic} on cue-induced reinstatement would be caused by non-specific behavioral suppression.

In contrast to cue-induced reinstatement, there was no effect of JD_{Tic} on stress-induced reinstatement of alcohol seeking, neither at the short nor the long pre-treatment interval. This contrasts with the previously reported finding that JD_{Tic} suppressed stress-induced reinstatement of cocaine seeking (Beardsley et al., 2005). This apparent discrepancy may be related to differences between neuroadaptive consequences of alcohol and cocaine self-administration, respectively. Alcohol is self-administered by genetically non-selected rats only at modest levels, and unless procedures that lead to higher levels of intoxication and dependence are imposed, alcohol self-administration does not lead to neuroadaptations that engage brain stress circuitry (Heilig and Koob, 2007). In contrast, cocaine is a potent reinforcer that is spontaneously administered at high rates, and is a potent stimulus for up-regulation of DYN expression (Hurd et al., 1992). Stress-induced reinstatement utilizes a single footshock stressor phase to reactivate lever responding, which may engage KOR signaling when the DYN/KOR system has been recruited through a history of cocaine self-administration, but not in the absence of such recruitment. While these results are somewhat unexpected, it is of note that naltrexone, which non-specifically blocks opioid receptors, suppresses reinstatement induced by cues, but not by stress (Liu and Weiss, 2002; Le et al., 1999), a behavioral profile similar to that of JD_{Tic}.

Due to the behavioral profile of JD_{Tic}, one concern is that this compound could be exerting its effects via inhibition of MOR signaling. The ability of KOR antagonists to block the MOR was first suggested in a study using mice (Broadbear et al., 1994), but has not been replicated in rats (Picker et al., 1996). Even though the evidence to support such a concern is rather thin, it is important to address this possible confound. To address such concerns we assessed tail flick nociception following morphine injection, and found that JD_{Tic} pretreatment had no effect on morphine-induced analgesia. This is supported in the literature by findings demonstrating that JD_{Tic} at a 1 h pretreatment time had no effect on tail flick analgesia following morphine treatment in mice (Jackson et al., 2010). In addition, we show here that JD_{Tic} at a 2 h pretreatment interval has no effect on locomotor activation induced by a non-maximal dose of morphine. Taken together, these findings suggest that JD_{Tic} does not impact signaling at the MOR. However, we cannot rule out the possibility that morphine activates the MOR in a different way than endogenous MOR ligands (Sauliere-Nzeh et al., 2010), and that JD_{Tic} antagonism may impact the latter and not the former. We suspect that any potential impairment of brain MORs would be indicated by our present experimental design, but this issue warrants further exploration. Further, the impact of JD_{Tic} on cue-induced reinstatement suggests that it is not exerting its behavioral effects via the MOR, as MOR specific antagonists have not been found to attenuate cue-induced reinstatement (Ciccocioppo et al., 2002; Marinelli et al., 2009).

Taken together, these results indicate a complex and state-dependent influence of KOR antagonists on alcohol related behaviors. The duration of JD_{Tic}'s behavioral effects are significantly different for anxiolysis versus suppression of self-administration and cue-induced reinstatement. These observations suggest a potential utility of KOR antagonists such as JD_{Tic} in treatment of alcohol dependence.

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Figure 1. JDTic reverses acute alcohol withdrawal-induced anxiety

Anxiogenic-like behavior induced by acute alcohol withdrawal was tested on the elevated plus-maze after pretreatment with JDTic or vehicle. Data are expressed as percentage of time spent exploring the open arms of the apparatus. There was a significant main effect of treatment, and post-hoc analysis showed a significant difference from the Vehicle - Saline group for both the Vehicle - Alcohol and JDTic 3 mg/kg - Alcohol groups, but not the JDTic 10 mg/kg - Alcohol group. Therefore, antagonism of the KOR by administration of JDTic is capable of reversing anxiety-like behavior in this model of “hangover anxiety”. * $p < 0.05$ compared to Vehicle-Saline group.

Figure 2. JDtic decreases alcohol self-administration

Alcohol self-administration was measured 2, 24, and 48h after a single injection of JDtic. There was a main effect of session, as well as a treatment x session interaction. Post-hoc analysis showed a significant difference from baseline responding only in the JDtic 10 mg/kg pretreated group at the 2h pretreatment time-point; also, at that time-point, the 10 mg/kg JDtic treated group differed from the vehicle group. These results indicate that JDtic is capable of decreasing alcohol self-administration in rats when given 2h prior to the session, but not at later time-points. # $p < 0.05$ compared to vehicle treated group for that treatment timepoint. ** $p < 0.01$ compared to baseline responding for that treatment group.

Figure 3. nor-BNI decreases alcohol self-administration

Alcohol self-administration was measured 2, 24, and 48 h after a single injection of nor-BNI (30 mg/kg). There was a main effect of treatment, as well as a treatment x session interaction. Post-hoc analysis showed a significant difference from baseline responding only in the nor-BNI pretreated group at the 2h pretreatment time-point; also, at that time-point, the nor-BNI treated group differed from the vehicle group. These results indicate that nor-BNI is capable of decreasing alcohol self-administration in rats when given 2h prior to the session, but not at later time-points. ## $p < 0.01$ compared to vehicle treated group for that treatment timepoint. ** $p < 0.01$ compared to baseline responding for that treatment group.

Figure 4. JDTic does not affect self-administration of 10% sucrose

Self-administration of 10% sucrose solution was measured after injection of JDTic or vehicle. Two way ANOVA indicated that there was no main effect of drug treatment or treatment x session interaction. These results indicate that JDTic had no effect on self-administration of a natural reward that may confound results of experiments measuring drug reinforcement using operant responding.

Figure 5. nor-BNI does not affect self-administration of 10% sucrose

Self-administration of 10% sucrose solution was measured after injection of nor-BNI (30 mg/kg) or vehicle. Two way ANOVA indicated that there was no main effect of drug treatment, session, or treatment x session interaction. These results indicate that nor-BNI had no effect on self-administration of a natural reward that may confound results of experiments measuring drug reinforcement using operant responding.

Figure 6. JDTC attenuates cue-induced reinstatement

Cue-induced reinstatement was detected as a significantly higher response rate on testing following exposure to cues that were previously paired with alcohol availability compared to extinction responding in the vehicle-treated group. There was a main effect of drug treatment on responses on the alcohol associated lever. Post hoc analysis showed that 2h pretreatment with JDTC significantly decreased reinstatement responding compared to both the vehicle treated group and the group treated with JDTC 24h prior to reinstatement testing. There was no effect of JDTC treatment on inactive lever responding. ## $p < 0.01$ compared to reinstatement responding in vehicle group. * $p < 0.05$ compared to reinstatement responding in both vehicle and 24h JDTC pretreatment groups.

Figure 7. JDTic does not affect stress-induced reinstatement

Stress induced reinstatement was detected as a significantly higher response rate on testing following exposure to intermittent footshock compared to extinction responding in the vehicle-treated group. There was no effect of drug treatment on responses on the alcohol associated lever. Responses on the inactive lever were not affected by drug treatment. Taken together, these results show that JDTic has no effect on stress-induced reinstatement of alcohol seeking. $##p < 0.01$ compared to reinstatement responding in the vehicle treated group.

Figure 8. JDTic specifically blocks the KOR at the 2h pretreatment time point

Tailflick nociception was measured following injection of morphine (10mg/kg) or U50488 (20 mg/kg) after pretreatment with JDTic (10 mg/kg) or saline. There was a significant effect of treatment. Post-hoc tests indicated that the Saline/U50488 and Saline/Morphine groups were significantly different from the Saline/Saline group, indicating that both morphine and U50488 produced analgesia. The JDTic/morphine group also differed significantly from the Saline/Saline group, indicating that JDTic did not affect the analgesic properties of a MOR agonist. The JDTic/Saline and JDTic/U50488 groups were not significantly different from the Saline/Saline group. These findings show that JDTic by itself has no analgesic properties, and that it is capable of blocking the analgesic effects of a KOR agonist. Taken together, these results demonstrate that JDTic is capable of blocking KORs at a short pretreatment time of 2 h, and that the inhibitory effects of JDTic at this timepoint are specific for KORs and do not extend to any action at the MOR. * $p < 0.05$ compared to saline/saline group.

Figure 9. JDITic does not affect locomotor activity

JDITic did not affect total locomotor activity over 1 h as measured by distance traveled (Figure 9A). When data were broken down into 10 min bins (Figure 9B), there was a main effect of bin, reflecting that rats introduced to the novel environment showed a high initial level of exploratory activity that gradually decreased over time as the animals habituated to the testing chambers. There was, however, no main effect of drug treatment or bin x treatment interaction. These results show that JDITic did not have any non-specific sedative effects that could account for suppression of lever pressing behavior, and also that it does not influence exploratory activity in a novel environment.

Figure 10. JDTic does not affect morphine-induced locomotor activation

JDTic, when administered 2 h prior to morphine injection, had no effect on morphine-induced locomotor activation. One way ANOVA revealed a significant main effect of treatment and post-hoc tests indicated that locomotion was elevated in both the vehicle/morphine and JDTic/morphine groups when compared to vehicle/vehicle treated rats. There was no significant difference between locomotion in the vehicle/morphine and JDTic/morphine groups. The lack of JDTic effect on a non-maximal dose of morphine in locomotor activity experiments further supports our interpretation that JDTic at 2 h after administration has no effect on MOR signaling. ** $p < 0.01$ compared to vehicle/vehicle group.