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ORIGINAL INVESTIGATION

Selective blockade of the orexin-2 receptor attenuates ethanol self-administration, place preference, and reinstatement

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Abstract
Rationale Orexin-1 receptor antagonists have been shown to block the reinforcing effects of drugs of abuse and food. However, whether blockade of orexin-2 receptor has similar effects has not been determined. We have recently described the in vitro and in vivo effects of JNJ-10397049, a selective and brain penetrant orexin-2 receptor antagonist.
Objective The goal of these studies was to evaluate whether systemic administration of JNJ-10397049 blocks the rewarding effects of ethanol and reverses ethanol withdrawal in rodents. As a comparison, SB-408124, a selective orexin-1 receptor antagonist, was also evaluated.
Methods Rats were trained to orally self-administer ethanol (8% *v/v*) or saccharin (0.1% *v/v*) under a fixed-ratio 3 schedule of reinforcement. A separate group of rats received a liquid diet of ethanol (8% *v/v*) and withdrawal signs were evaluated 4 h after ethanol discontinuation. In addition, ethanol-induced increases in extracellular dopamine levels in the nucleus accumbens were tested. In separate experiments, the acquisition, expression, and reinstatement of conditioned place preference (CPP) were evaluated in mice.
Results Our results indicate that JNJ-10397049 (1, 3, and 10 mg/kg, sc) dose-dependently reduced ethanol self-administration without changing saccharin self-administration, dopamine levels, or withdrawal signs in rats. Treatment with JNJ-10397049 (10 mg/kg, sc) attenuated the acquisition, expression, and reinstatement of ethanol CPP and ethanol-induced hyperactivity in mice. Surprisingly, SB-408124 (3, 10 and 30 mg/kg, sc) did not have any effect in these procedures.
Conclusions Collectively, these results indicate, for the first time, that blockade of orexin-2 receptors is effective in reducing the reinforcing effects of ethanol.

Keywords Hypocretin · Alcoholism · Self administration · Microdialysis · Reward · Orexin receptor type 2

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Introduction

The orexin (or hypocretin) system consists of two neuropeptides, orexins-A and -B, which are produced almost exclusively in the lateral hypothalamus (de Lecea et al. 1998; Sakurai et al. 1998) and project to a wide array of discrete brain regions (Peyron et al. 1998). The orexin peptides bind to two orexin receptors, the orexin-1 and -2 receptors. Orexin-B binds with similar affinity to both orexin receptors, while orexin-A has a 20–30-fold-higher affinity for the orexin-1 receptor (Sakurai et al. 1998). The orexins were termed for their initially discovered feeding effect (Sakurai et al. 1998), and were soon after implicated in narcolepsy (Chemelli et al. 1999; Lin et al. 1999), which initiated an intense research effort in the field. Indeed, a vital function for the orexin system in arousal and sleep/wake states has clearly been established (for a review, see Ohno and Sakurai 2008). However, the lateral hypothalamic location of the orexins, their projections to brain areas involved in emotional processing, and their role in feeding would also suggest a possible involvement in appetitive

behaviors, motivation, or reward, and thereby addiction. The first suggestion of an interaction between the orexin system and drugs of abuse were findings of an alteration to the orexin system after nicotine treatment (Kane et al. 2000, 2001) and altered morphine withdrawal in prepro-orexin knockout mice (Georgescu et al. 2003). More recent research, focusing on animal models of drug intake, reward, and reinstatement, has expanded our knowledge of the orexin system as it relates to addiction. A comprehensive set of data now suggest that drugs of abuse activate the orexin system, which in turn enhances drug reward or drug seeking (for a review, see Aston-Jones et al. 2009), thus presenting the exciting possibility of treating addiction with novel therapeutic interventions that target the orexin system.

Most orexin-addiction research has focused on elucidating the function of the orexin-1 receptor. It has been shown that the orexin-1 receptor antagonist SB-334867 attenuates morphine-conditioned place preference (CPP) and reinstatement (Harris et al. 2005, 2007; Narita et al. 2006), stress-induced cocaine reinstatement (Boutrel et al. 2005); but see lack of effect of intra-VTA orexin-1 receptor blockade on stress-induced cocaine reinstatement (Wang et al. 2009), cue-induced cocaine reinstatement (Smith et al. 2009), cocaine-induced synaptic plasticity and sensitization (Borgland et al. 2006), and intake and cue- and stress-induced reinstatement of ethanol (Lawrence et al. 2006; Richards et al. 2008; Sakurai et al. 1998). In addition, SB-334867 attenuates precipitated morphine withdrawal (Sharf et al. 2008) and nicotine self-administration (Hollander et al. 2008).

Due to a previous lack of publicly available selective orexin-2 receptor antagonists, very little is known about the specific role of the orexin-2 receptor in addiction, although it has recently been reported that an orexin-2 receptor antagonist does not affect cocaine reinstatement or intake (Smith et al. 2009). Since orexins-A and -B, which have similar affinities for orexin-2, but different affinities for orexin-1 (Sakurai et al. 1998), evoke similar increases in nucleus accumbens dopamine (DA) levels and produce similar CPP at similar doses (Narita et al. 2007), this suggests that the orexin-2 receptor may also be involved in reward pathways. A recent study substantiates this hypothesis, reporting that the effects of orexin-A on dopaminergic cell firing were only partially blocked by an orexin-1 receptor antagonist, but fully blocked by an orexin-2 receptor antagonist (Malherbe et al. 2009).

Recently, we described a novel orexin-2 receptor antagonist, JNJ-10397049, which has good brain exposure following systemic injection and high affinity and selectivity for the orexin-2 receptor (Dugovic et al. 2009). The aim of the present study was to test JNJ-10397049 in the oral ethanol self-administration and conditioned place preference model, in rats and mice, respectively. Furthermore, since an ideal treatment for ethanol addiction would also prevent relapse and attenuate withdrawal, we also examined reinstatement of place preference in mice and ethanol withdrawal signs in rats. Finally, we also measured ethanol-induced increases in dopamine by microdialysis. Ethanol is believed to mediate its rewarding or addictive effects, at least in part, by stimulation of dopamine in the nucleus accumbens (Imperato and Di Chiara 1986; Pfeffer and Samson 1988), and the orexins have been shown to modulate DA cell firing and DA extracellular levels (Baldo et al. 2003; Malherbe et al. 2009; Narita et al. 2007). In addition to testing the orexin-2 receptor antagonist, we also chose to test SB-408124, a selective orexin-1 receptor antagonist which has been described in the literature. SB-408124 has similar affinity and selectivity for the orexin-1 receptor as SB-334867, but also has faster clearance (Upton 2005), and reaches full orexin-1 receptor occupancy in rats after 30 mg/kg s.c., which results in brain levels of only about 1 μM (Dugovic et al. 2009). SB-408124 has less exposure in the mouse, but still reaches a maximum exposure of 0.5 μM in the brain, with stable levels from 0–60 min, after 30 mg/kg s.c. (unpublished data).

Materials and methods

Subjects

For the CPP studies, male DBA/2 mice (Jackson Labs), 8–12 weeks old were used. For the self-administration, microdialysis, and ethanol withdrawal studies, male Wistar rats (Charles River), weighing 200–320 g, were used.

Subjects were housed on a normal 12 h light–dark cycle (lights on at 06:00) with food and water (or a liquid diet, in the case of the withdrawal studies) provided ad libitum. Experiments took place during the light cycle between 08:00 and 15:00. All methods complied with *The Guide for the Care and Use of Laboratory Animals* manual and were approved by the IACUC.

Oral ethanol and saccharin self-administration

Self-administration experiments were conducted in sound attenuated operant chambers (Med-Associates, St Albans, VT, USA) equipped with one house light, two retractable levers, two stimulus lights located above the levers, and two liquid hoppers.

Sixteen rats were used in this study. Sessions started with the illumination of the left and right light and lasted for up to 30 min or until 100 saccharin rewards were delivered, whichever occurred first. Initially, all rats were trained to lever press under a fixed-ratio (FR) 1 schedule of reinforcement. Pressing the active lever, which was assigned equally right and left, resulted in the delivery of

0.1 ml of a liquid solution of saccharin (0.1% *v*/*v*). Pressing the inactive lever had no programmed consequence but was recorded. When the number of saccharin deliveries was greater than 50 the FR was increased to 2 and, following the same criterion, to a final FR3. When the number of saccharin deliveries was stable for three consecutive sessions (did not change by more than 20%), half of the animals were switched to a mixed liquid solution of saccharin (0.1% *v*/*v*) and ethanol (8% *v*/*v*) and, upon reaching stability, to a 0.1 ml solution of ethanol (8% *v*/*v*) only. The remaining half continued to respond for saccharin (0.1% *v*/*v*) only. Tests began when stability was reached under the final schedule of reinforcement. Specifically, JNJ-10397049 (15 min pretreatment time at 1, 3, and 10 mg/kg, s.c.), SB-408124 (0 min pretreatment time at 3, 10, and 30 mg/kg, s.c.), or their respective vehicle was administered using a Latin-square design. In addition, animals received naltrexone (30 min pretreatment time at 1 mg/kg, s.c.) as a positive control.

CPP testing apparatus

An un-biased, two-chamber, custom-made CPP apparatus was used. The apparatus, made of plexiglass, measured 35.6×20.3×35.6 cm (*L*×*W*×*H*) and was separated into two equally sized chambers by a wall that ran along the width. The wall contained a door (7.6×7.6×7.6 cm) that could be opened or closed. The drug-paired chamber was patterned with white and black checkers and contained a piece of filter paper (placed out of reach of the animal) scented with 200 μL of almond extract (Kroger Co., Cincinnati, Ohio). The opposite chamber contained a similar amount of white and black, in a cow-print pattern, and a piece of filter paper scented with 200 μl of lemon extract (Kroger Co., Cincinnati, Ohio). The apparatus was lined with eight photobeams (Kinder Scientific, San Diego, CA) along its length and four along its width, evenly spaced, to determine the position and locomotor activity (measured as total cm of distance traveled) of the animal.

Acquisition of ethanol CPP and ethanol-induced hyperactivity during conditioning

The methods used were similar to published methods (Cunningham et al. 1992). On day 1, animals were placed in the apparatus for 15 min with free access to both chambers as a habituation, and the time spent in each chamber was recorded. On days 2–11, on alternating days, animals were injected with vehicle (water; 12.5 ml/kg) and confined to the cow-print chamber, or injected with ethanol (2 g/kg, i.p.) and confined to the opposite chamber, for 5 min. To determine the effects of JNJ-10397049 and SB-408124 on the acquisition of ethanol CPP, JNJ-10397049 (10 mg/kg, s.c.), SB-408124 (30 mg/kg, s.c.), or vehicle was injected 1 h before ethanol (2 g/kg, i.p.) conditioning, with only vehicle injected 1 h before vehicle conditioning. An additional group of control animals were pretreated with only vehicle and conditioned to only the ethanol vehicle in each chamber. Locomotor activity was recorded during each of these five vehicle and five drug conditioning sessions to determine the effects of JNJ-10397049 and SB-408124 on ethanol-induced hyperactivity. On day 13, animals were placed into the apparatus in a drug-free state for 15 min, with free access to both chambers, and the time spent in each chamber was recorded as a test for CPP. In a separate study, to determine if the effects of JNJ-10397049 on ethanol CPP were due to intrinsic aversive properties, a separate group of animals were conditioned to JNJ-10397049 (10 mg/kg, s.c.) or its vehicle, without ethanol, using the same procedure as above.

Expression of ethanol CPP

A separate group of animals were conditioned to 2 g/kg ethanol as described above and pretreated with JNJ-10397049 (10 mg/kg, s.c.) or vehicle 1 h before, or SB-408124 (30 mg/kg, s.c.) or vehicle 30 min before, a 15 min CPP test to determine the effects of the orexin antagonists on the expression of an already-formed ethanol CPP.

Reinstatement of ethanol CPP

A separate group of animals were conditioned to 2 g/kg ethanol as described above, given a 15 min CPP test, and then exposed daily to the CPP apparatus, with free access to both chambers and without vehicle or ethanol injections, for 30 min a day to extinguish that CPP. Once the CPP was extinguished (the mean significantly less than the CPP test for two consecutive days), animals were assigned to three treatment groups, which were balanced for time spent in the drug-paired chamber during habituation, the CPP test, and the last extinction day. Animals were treated with JNJ-10397049 (10 mg/kg, s.c.) or vehicle 1 h before receiving a priming injection of ethanol (1 g/kg, i.p.), and then were immediately placed in the apparatus for another 15 min test of CPP.

Ethanol withdrawal

Ethanol dependence was induced by providing animals with a liquid diet containing ethanol (8% *v*/*v*). Initially, rats had access to a liquid nutritionally complete and calorically balanced diet (0% ethanol diet). The diet (Research Diets Inc, New Brunswick, NJ, USA) was in the form of a powder premix, which contains proteins (49 kcal%), carbohydrate (24 kcal%) and fat (27 kcal%), and was

prepared fresh every 24–72 h by mixing it with potable water. For the 0% ethanol diet, sucrose and corn starch were also added. After 1 week, rats received the diet mixed with a fixed percentage of ethanol (8% *v*/*v*), by substituting the sucrose and corn starch with ethanol to obtain the same kcal as the 0% alcohol diet. The liquid diet was the rat's only food source, but provided a complete and calorically balanced food source. Rats were then maintained on this ethanol-containing liquid diet for at least 2 weeks and until a stable baseline of intake was obtained. Our data indicated that the rats on the forced ethanol diet consumed an average of 9 g/kg/day of ethanol. Although we did not measure the blood ethanol content of the animals, others have found that an 8% ethanol diet results in blood ethanol levels of about 100 mg/dl and induces withdrawal and anxiety when the ethanol is withheld after chronic consumption (Knapp et al. 2005; Meert 1993). Our data indicated that clearly at least some level of dependence was achieved, since the animals displayed signs of physical withdrawal when the ethanol was withheld.

Tests were conducted once or twice per week when intake was stable (did not vary by more than 20% of baseline). On test day, ethanol deprivation was initiated by substituting the ethanol-liquid diet (8% *v*/*v*) with an ethanol-free liquid diet (0% liquid diet, with sucrose and corn starch substituted for the ethanol to maintain caloric level). The following signs of withdrawal were observed and recorded using a visual rating scale: tremors, piloerection, muscle rigidity, and vocalization. Specifically, the absence of a withdrawal sign was recorded as 0, whereas the presence was recorded as 1. Recording was conducted twice. First, animals were observed for 15 s while they were freely moving in the cage, then again for an additional 15 s while they were handled. Thus, the withdrawal sign rating scale ranged from 0 (no signs) to 8 (all four signs were present during both ratings). These measurements were taken at 4 h after removal of the ethanol-liquid diet (this time point was chosen as the peak of withdrawal based on our preliminary studies) and also before removal of the ethanol-liquid diet as a baseline measurement. The pretreatment time for diazepam (0.3, 1, and 3 mg/kg, s.c.) and its vehicle was 30 min. The pretreatment time for JNJ-10397049 (1, 3, and 10 mg/kg, s.c.), SB-408124 (3, 10, and 30 mg/kg, s.c.), and their respective vehicles was 15 min. Experiments were conducted using a Latin-square design.

Microdialysis

Microdialysis experiments were performed as previously described (Dugovic et al. 2009) using a guide cannula (Eicom, Kyoto, Japan) in the nucleus accumbens core (incisor bar, −3.5 mm, +1.8 mm anterior, 1.6 mm lateral, and 6.0 mm ventral to Bregma; Paxinos and Watson 1997). Microdialysis probes (2 mm active membrane length, Eicom) were perfused with artificial cerebral spinal fluid (147 mM NaCl, 4.0 mM KCl, 0.85 mM $MgCl_2$, 2.3 mM $CaCl_2$) at a rate of 1 μl/min and implanted the afternoon prior to sample collection. The following morning, six 10-min baseline samples were collected prior to drug injections. Separate groups of animals were then pretreated with JNJ-10397049 (10 mg/kg, s.c.), SB-408124 (30 mg/kg, s.c.), or their respective vehicle, immediately before an injection of ethanol or its vehicle. Ethanol was diluted to 20% with sterile water and delivered at 15.7 ml/kg (i.p.) for a dose of 2.5 g/kg. Samples were collected every 10 min for 2-h post dosing into a 96-well plate maintained at 4°C containing 2.5 μl of antioxidant (0.1 M acetic acid, 1 mM oxalic acid, and 3 mM L-cysteine in ultra pure water). Microdialysis samples were analyzed for DA by HPLC with electrochemical detection (Eicom) as previously described (Barbier et al. 2007; Bonaventure et al. 2007).

Motor performance

In a follow-up study, to determine if JNJ-10397049 interacted with ethanol to produce sedative effects that would have interfered with operant performance, a separate group of rats were tested on the rotarod (Stoelting, Wood Dale, IL). Briefly, rats were trained the day before the test to walk on the rotarod for 60 s. Rats that failed to meet this cutoff criteria of 60 s for at least three trials (out of a maximum of eight trials) were excluded from the study. On the following day, animals were co-injected with vehicle or 10 mg/kg JNJ-1039049 and 0, 1, or 2 g/kg ethanol, and 15 min later tested on the rotarod with a single trial. A maximum cutoff time of 60 s was used and 60 s was averaged in for rats meeting this cutoff. The data from the rotarod experiment were analyzed using nonparametric statistics due the non-normal distribution arising from the imposed cutoff. The data were analyzed with a Kruskal–Wallis ANOVA followed by Mann–Whitney post-hoc tests with a Bonferroni correction for multiple comparisons, comparing JNJ-10397049 to vehicle within each ethanol group to determine if JNJ-10397049 potentiated ethanol's sedative effects, and comparing the two doses of ethanol (pooled between JNJ-10397049 and vehicle) to its vehicle group to determine if there was a main effect of ethanol in producing sedation.

Pharmacological treatments

JNJ-10397049 (1-(2,4-dibromophenyl)-3-[(4*S*,5*S*)-2,2-dimethyl-4-phenyl-1,3-dioxan-5-yl]urea) was formulated in 5% Pharmasolve, 20% Solutol, 15% hydroxylpropyl-beta-cyclodextrin and administered at 10 ml/kg to mice and 1 ml/kg to rats. SB-408124 (1-(6,8-difluoro-2-methylquinolin-4-yl)-3-[4-(dimethylamino)phenyl]urea) was formulated in 30% sulphobutylether-beta-cyclodextrin at

2 ml/kg for rats and in 5% Pharmasolve, 20% Solutol, 15% hydroxylpropyl-beta-cyclodextrin at 10 ml/kg in mice. Naltrexone was dissolved in saline and administered at 1 ml/kg. Diazepam was dissolved in 5% dimethylsulfoxide, 20% Cremophor, 75% water and administered at 1 ml/kg. JNJ-10397049 was synthesized in house, and SB-408124 was either synthesized in house or purchased from Tocris (Ellisville, MO, USA). Naltrexone and diazepam were purchased from Sigma-Aldrich (St Louis, MO, USA). The doses and pretreatment times of JNJ-10397049 and SB-408124 were based on their pharmacokinetics and ex vivo receptor binding studies (Dugovic et al. 2009).

Data analysis

Self-administration of ethanol and saccharin were measured as total number of lever presses, using a one-way repeated measures ANOVA for dose and Duncan post-hoc tests when appropriate. One-tailed tests were used, since only decreases in operant responding would indicate efficacy. The acquisition of CPP was analyzed for % time in the drug-paired chamber using a two-way analysis of variance (ANOVA) for treatment and test (pre- versus post-conditioning), with repeated measures on test. Ethanol hyperactivity was measured using a three-way ANOVA for conditioning session×conditioning stimulus×treatment, with repeated measures on session and stimulus, for total distance traveled (cm). Expression and reinstatement was analyzed for % time in the drug-paired chamber using a two-way ANOVA for treatment and test (either expression versus pre-conditioning, or reinstatement versus extinction), with repeated measures on test. Duncan's post-hoc tests were used to detect significant differences, when appropriate. One-tailed tests were used for the CPP tests, since the CPP test was used to only measure increases in time (preference) or increases in activity (hyperactivity). Microdialysis samples were analyzed as % baseline with a two-tailed three-way ANOVA for pretreatment, treatment, and time, with repeated measures on time, and two-tailed Duncan's post-hoc tests. Signs of ethanol withdrawal were analyzed using a two-tailed Kruskal–Wallis ANOVA and two-tailed Mann–Whitney *U* tests with Bonferroni corrections as post-hoc tests when appropriate. Data was analyzed using Graphpad Prism (San Diego, CA) or Statistica (Tulsa, OK) software.

Results

Ethanol and saccharin self-administration

A one-way repeated measures ANOVA revealed a significant effect of dose on ethanol responding (Fig. 1a; $F(3,15)=3.14$, $p=0.03$; $N=6$/group) and further post-hoc analysis revealed that JNJ-10397049 significantly decreased ethanol self-administration (total lever presses) at the doses of 3 and 10 mg/kg ($p=0.03$ and $p=0.009$, respectively). JNJ-10397049 had no significant effect on the self-administration of saccharin (Fig. 1b; $F(3,21)=1.20$, $p=0.17$; $N=8$/group). In contrast, as shown in Figs. 1c and d, SB-408124 (3–30 mg/kg) had no effect on the number of total lever presses for either ethanol ($F(3,15)=1.39$, $p=0.14$; $N=6$/group) or saccharin ($F(3,21)=1.15$, $p=0.18$; $N=8$/group). The positive control naltrexone (1 mg/kg) decreased ethanol self-administration (Fig. 1e; $F(6)=4.72$, $p=0.002$; $N=7$/group) and also decreased saccharin self-administration (Fig. 1f; $F(5)=2.65$, $p=0.02$; $N=6$/group).

Pre-conditioning

During the habituation test, animals spent 48.6% of their time in the checker patterned chamber, which was not significantly different from the hypothetical mean of 50%, which represents no bias for either chamber (one-sample *t* test), indicating that the procedure used was balanced.

Acquisition of CPP

Analyzing %time spent in the drug-paired chamber during the ethanol CPP test revealed a significant treatment×test interaction (Fig. 2a; $F(3, 38)=2.68$, $p=0.03$; $N=12$ for vehicle+vehicle, $N=11$ for vehicle+ethanol, $N=10$ for JNJ-10397049+ethanol, $N=9$ for SB-408124+ethanol). Post-hoc tests demonstrated that the vehicle-pretreated, ethanol-conditioned animals displayed a significant place preference compared to the vehicle-pretreated, vehicle-conditioned animals ($p=0.03$) and compared to their pre-conditioning time ($p=0.04$). Animals pretreated with SB-408124 (30 mg/kg) and conditioned to ethanol likewise displayed a significant place preference compared to controls ($p=0.001$) and pre-conditioning time ($p=0.005$). JNJ-10397049 (10 mg/kg) pretreated animals, however, did not display a significant place preference compared to controls ($p=0.23$) and did not change from pre-conditioning time ($p=0.48$). To determine if the effects of JNJ-10397049 on ethanol CPP were due to intrinsic aversive properties, JNJ-10397049 was conditioned by itself using the same procedure. JNJ-10397049 did not differ from its vehicle in terms of %time spent in the drug-paired chamber (Fig. 2b; $F(1, 21)=0.02$, $p=0.45$; $N=12$ for vehicle, $N=11$ for JNJ-10397049), indicating that JNJ-10397049 is void of motivational properties per se. JNJ-10397049 also did not differ from its vehicle in terms of locomotor activity during conditioning ($F(1, 21)=1.31$, $p=0.26$, main effect of treatment; $F(4, 84)=1.45$, $p=0.22$, day×treatment interaction; $F(1, 21)=1.70$, $p=0.21$, conditioning session×treatment interaction; $F(4, 84)=1.10$, $p=0.36$, day×

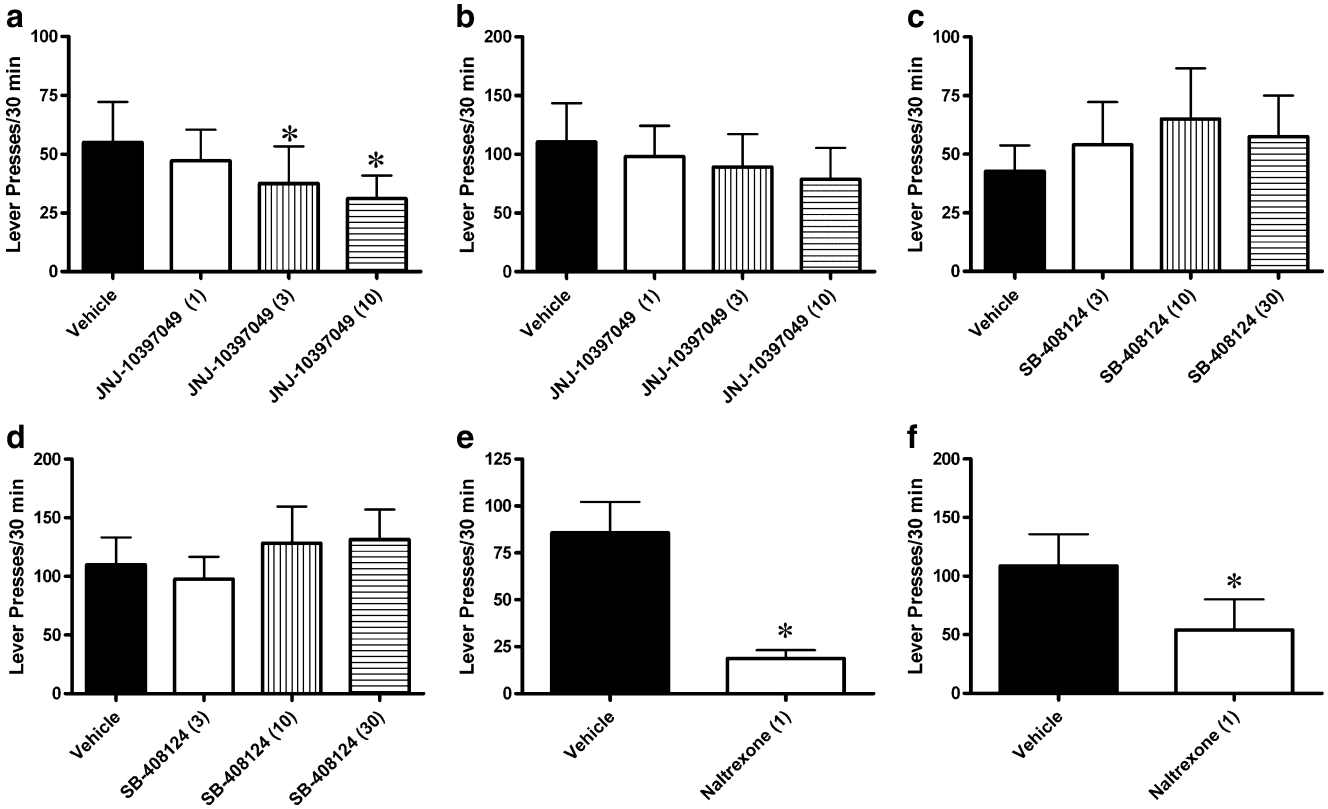

Fig. 1 The selective orexin-2 antagonist, JNJ-10397049, decreased lever pressing for ethanol at the doses of 3 and 10 mg/kg (**a**), without significantly affecting lever pressing for saccharin (**b**), indicating that the effects were selective and not due locomotor impairments or a general disruption to motivation. The selective orexin-1 antagonist, SB-408124, had no significant effects on ethanol (**c**) or saccharin (**d**) self-administration. Naltrexone significantly decreased operant responding for ethanol (**d**), to a greater magnitude than JNJ-10397049, but also decreased responding for saccharin (**e**). *$p<0.05$ compared to vehicle. Doses (mg/kg) are given in *parentheses*. *N*'s are six to eight per group

conditioning session×treatment interaction). On average, animals had a distance traveled of 93±11 cm after vehicle treatment and 58±12 cm after JNJ-10397049 treatment.

Ethanol hyperactivity

In analyzing the locomotor activity data from the mice undergoing acquisition of ethanol CPP, the ANOVA revealed a significant conditioning stimulus×treatment interaction (Fig. 2c; $F(3,38)=2.27$, $p=0.047$), and the post-hoc test revealed that ethanol significantly stimulated locomotor activity in vehicle-pretreated animals during conditioning ($p=0.03$ comparing vehicle-pretreated, ethanol-conditioned animals to vehicle-pretreated, vehicle-conditioned animals during drug conditioning sessions, and $p=0.002$ comparing drug conditioning and control conditioning sessions in vehicle-pretreated, ethanol-conditioned animals). SB-408124 did not attenuate ethanol-induced locomotor activity ($p=0.005$ compared to control animals during drug conditioning sessions, and $p=0.001$ comparing drug and vehicle conditioning sessions). JNJ-10397049 pretreated mice did not, however, display ethanol-induced hyperactivity ($p=0.26$ compared to control animals and $p=0.12$ comparing drug and vehicle conditioning sessions).

Expression of ethanol CPP

Animals that were conditioned to ethanol in the absence of the orexin antagonists were tested to determine if JNJ-10397049 (10 mg/kg) or SB-408124 (30 mg/kg) would block the expression of an already-formed ethanol CPP. JNJ-10397049 tended to attenuate the expression of ethanol CPP (Fig. 3a; $F(1,28)=2.00$, $p=0.08$, treatment×test interaction; 66.7±10.31% in the vehicle group ($N=16$) and 49.4±11.0% in the JNJ-10397049 group ($N=14$) during the expression test). JNJ-10397049 did not alter locomotor activity during the expression test. Vehicle treated animals had a distance traveled of 553±102 cm whereas JNJ-10397049 treated animals had a distance traveled of 387±109 cm ($F(1,28)=1.23$, $p=0.28$). For SB-408124, there was no such trend on expression of ethanol CPP (Fig. 3b; $F(1,20)=0.20$, $p=0.4$; $N=11$/group) and both the vehicle group and SB-408124 treated group spent over 60% of their time in the drug-paired chamber.

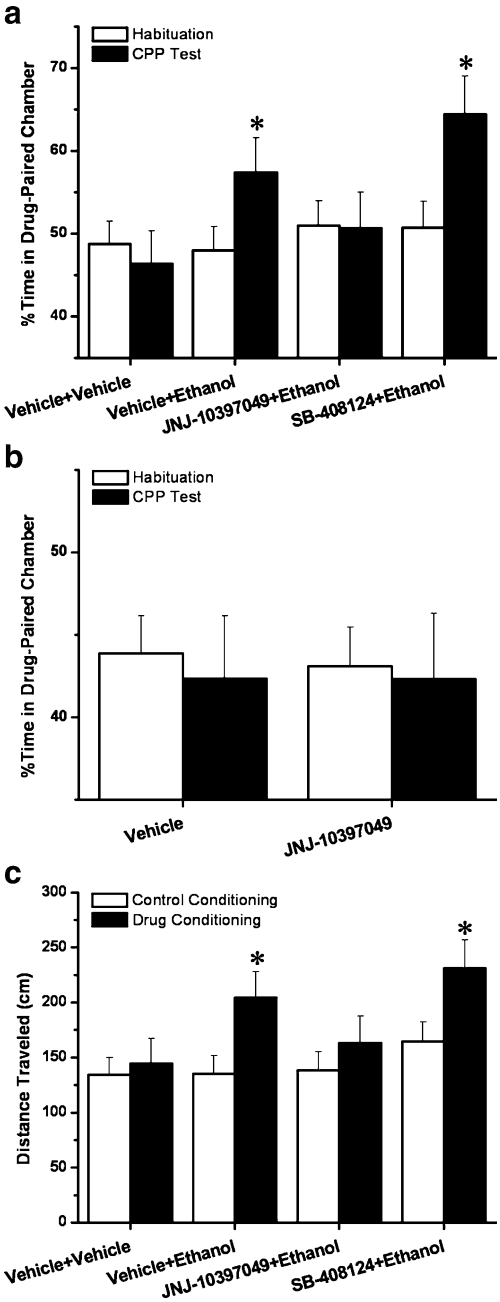

Fig. 2 **a** Animals conditioned to ethanol and pretreated with vehicle or SB-408124 (30 mg/kg) displayed a significant place preference compared to animals pretreated with vehicle and conditioned to vehicle. Animals pretreated with the selective orexin-2 antagonist JNJ-10397049 (10 mg/kg) before ethanol conditioning failed to acquire a significant place preference. **b** The effect of JNJ-10397049 (10 mg/kg) on the acquisition of ethanol place preference was not due to intrinsic aversive properties. JNJ-10397049 produced neither a place aversion nor place preference on its own. **c** Ethanol significantly increased locomotor activity compared to control animals. Pretreatment with JNJ-10397049 (10 mg/kg), but not SB-408124 (30 mg/kg), blocked ethanol-induced hyperactivity, so that the animals treated with JNJ-10397049 and ethanol had similar activity as control animals. *$p<$ 0.05 compared to vehicle+vehicle. N's are 9–12 per group

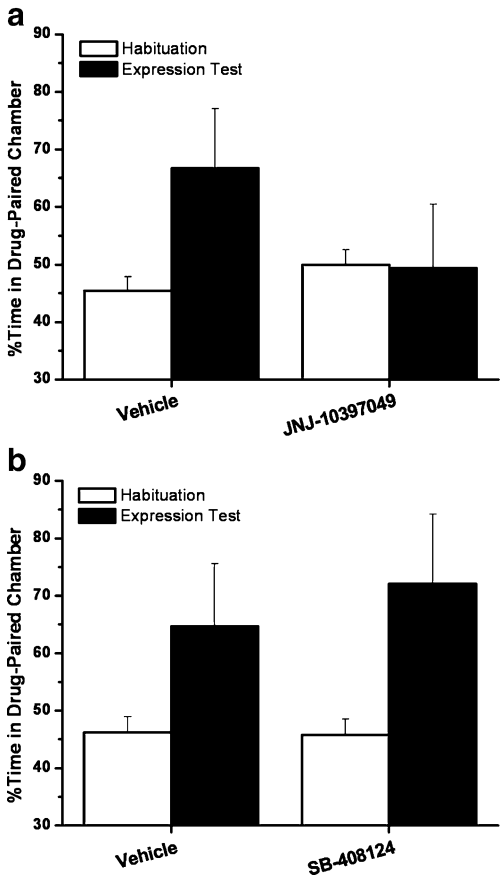

Fig. 3 When treated with JNJ-10397049 (10 mg/kg; **a**) or SB-408124 (30 mg/kg; **b**), or their respective vehicles, before a place preference test, JNJ-10397049 strongly tended to attenuate the expression of an already established ethanol place preference ($p=0.08$), whereas SB-408124 was clearly without effect. N's are 11–16 per group

Reinstatement of ethanol CPP

Vehicle-pretreated animals primed with an ethanol injection reinstated ($F(1, 19)=5.59$, $p=0.01$, group×test interaction; $p=0.02$ comparing the extinction and reinstatement tests in vehicle-pretreated animals, post-hoc test; $N=10$). As shown in Fig. 4, JNJ-10397049 (10 mg/kg) pretreatment before the prime significantly attenuated reinstatement ($p=0.007$, post-hoc test comparing the vehicle and JNJ-10397049 groups during the reinstatement test and $p=0.14$ comparing the extinction and reinstatement tests in JNJ-10397049 pretreated animals; $N=11$).

Acute ethanol withdrawal

Neither JNJ-10397049 (1–10 mg/kg) nor SB-408124 (3–30 mg/kg) had any effect on signs of ethanol withdrawal (Fig. 5a; $H(3,32)=1.47$, $p=0.69$; Kruskal–Wallis for JNJ-10397049; Fig. 5b; $H(3,32)=0.26$, $p=0.97$; Kruskal–Wallis for SB-408124; $N=8$/group). In contrast, the positive control diazepam significantly attenuated signs of ethanol

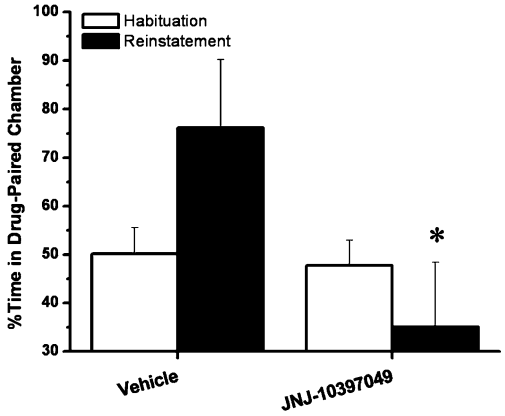

Fig. 4 Animals were conditioned to ethanol, tested for place preference, and then extinguished. In vehicle-pretreated animals, a priming injection of ethanol (1 g/kg) resulted in a significant, robust reinstatement of place preference. Animals pretreated with JNJ-10397049 (10 mg/kg) failed to reinstate following a priming injection. *$p<0.05$ compared to vehicle. *N*'s are 10–11 per group

withdrawal (Fig. 5c; H(3,31)=11.64, $p=0.009$; Kruskal–Wallis; $p=0.009$ comparing 3 mg/kg and vehicle, Mann–Whitney *U* test with Bonferroni correction; $N=8$/group).

Extracellular levels of dopamine in the nucleus accumbens

Absolute basal levels of DA in the dialysate (without adjusting for probe recovery) was 0.473±0.019 pg/μl. In the JNJ-10397049 experiment, animals treated with ethanol showed significant increases in extracellular DA levels compared to animals treated with the ethanol vehicle (Fig. 6a; $F(17,272)=5.84$, $p=0.000001$, treatment×time interaction; $p=0.00005$–$p=0.01$ at each time point from 20–120 min post-injection, post-hoc test; $N=5$ for vehicle+vehicle, 5 for vehicle+ethanol, 7 for JNJ-10397049+vehicle, and 6 for JNJ-10397049+ethanol). There was no effect of pretreatment or interactions with pretreatment, indicating that JNJ-10397049 (10 mg/kg) had no effects on either basal or ethanol-induced increases in dopamine levels ($F(1,19)=0.0049$, $p=0.94$, main effect of pretreatment; $F(1,19)=0.024$, $p=0.89$, pretreatment×treatment interaction;

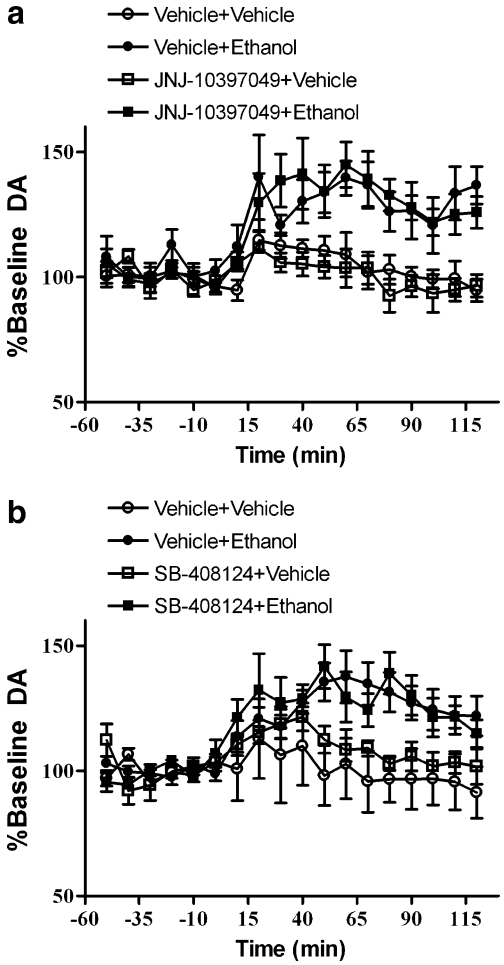

Fig. 6 Ethanol significantly increased DA release in the nucleus accumbens ($p<0.05$, **a** and **b**). Pretreatment with JNJ-10397049 (10 mg/kg; **a**) or SB-408124 (30 mg/kg; **b**) had no effect on basal DA release or on ethanol-induced DA release. *N*'s are five to seven per group

$F(17,323)=0.64$, $p=0.86$, pretreatment×time interaction; $F(17,323)=1.076$, $p=0.37$, pretreatment×treatment×time interaction). Likewise, in the SB-408124 experiment, animals treated with ethanol showed significant increases in DA levels (Fig. 6b; $F(17,306)=4.34$, $p=0.00001$, treatment×time interaction; $p=0.00003$ to $p=0.02$ from 50–120 min,

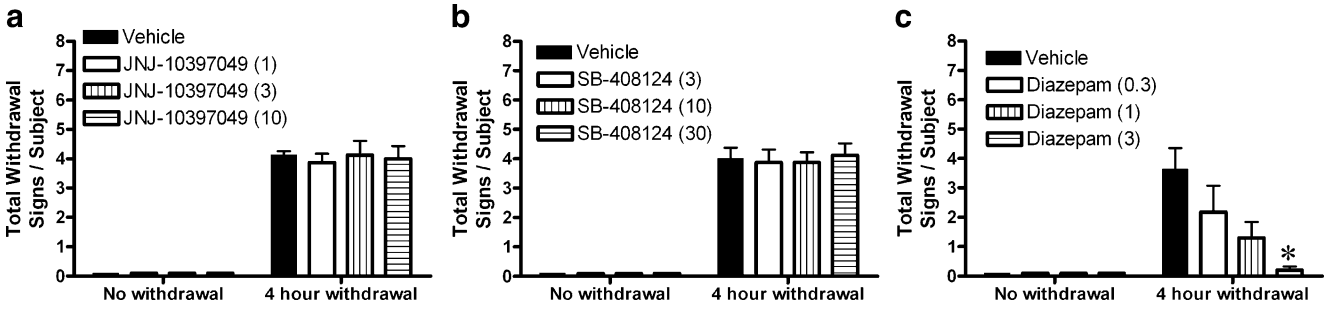

Fig. 5 Discontinuation of a forced liquid ethanol diet for 4 h resulted in robust withdrawal signs. Pretreatment with JNJ-10397049 (**a**) or SB-408124 (**b**) had no effect on signs of ethanol withdrawal, whereas the positive control diazepam significantly decreased ethanol withdrawal (**c**). Doses (mg/kg) are given in parentheses. *N*'s are eight per group

post-hoc test; N=5 for vehicle+vehicle, N=5 for vehicle+ethanol, N=5 for SB-408124+vehicle, N=7 for SB-408124+ethanol), and SB-408124 (30 mg/kg) was without effect on either basal or ethanol-induced DA increases in DA levels ($F(1,18)=0.57$, $p=0.46$, main effect of pretreatment; $F(1,18)=0.26$, $p=0.62$, pretreatment×treatment interaction; $F(17,306)=1.02$, $p=0.44$, pretreatment×time interaction; $F(17,306)=0.81$, $p=0.68$, pretreatment×treatment×time interaction).

Motor performance

The Kruskal–Wallis ANOVA revealed a significant difference between the six groups (Fig. 7; $H(5,N=64)=30.97$, $p=0.00001$). Post-hoc tests revealed that JNJ-10397049 produced no impairment at any ethanol dose compared to control animals at the same dose of ethanol ($p=2.69$, 2.16, and 2.50 at the doses of 0, 1, and 2 g/kg of ethanol, respectively, or $p=0.99$, 0.72, and 0.83 for the uncorrected values). However, ethanol by itself did produce a significant impairment in performance at the dose of 2 g/kg ($H(2,N=64)=30.45$, $p=0.00001$, Kruskal–Wallis test; $p=2.0$ and 0.0002 for 1 and 2 g/kg compared to vehicle, respectively, or $p=1.0$ and 0.00009 for the uncorrected values).

Discussion

The goal of these studies was to evaluate the effects of a selective orexin-2 receptor antagonist in animal models of alcohol abuse and dependence. Our results demonstrate for the first time that orexin-2 receptors are involved in mediating the reinforcing effects of alcohol, and that blockade of orexin-2 receptors attenuate alcohol reward.

We evaluated the effects of the orexin-2 receptor antagonist on ethanol self-administration in rats. Our data indicate that JNJ-10397049 dose-dependently reduced ethanol-reinforced lever presses at the doses of 3 and 10 mg/kg, but did not alter saccharin self-administration. These results suggest that the effects of JNJ-10397049 were selective for ethanol. Consistent with this, JNJ-10397049 produced no impairments on rotarod performance and did not potentiate the impairments produced by ethanol. It is indeed interesting that the orexin-2 antagonist did not decrease lever presses for saccharin or produce motor impairments on the rotarod, given that orexin-2 receptor antagonists induce sleep (Dugovic et al. 2009), and may be expected to decrease operant behavior by inducing sleep. This suggests that doses of orexin-2 receptor antagonists that are effective at promoting sleep do not prevent animals from completing challenging tasks when motivated to do so. These results suggest that the night time sleep inducing and maintaining effects of orexin-2 receptor antagonists may not disrupt daily activities during waking in humans, a substantial improvement over traditional sleep agents that target GABA-A receptors (Otmani et al. 2008). If orexin-2 receptor antagonists do allow subjects to remain awake when motivated to do so, then the sleep promoting effects of orexin-2 receptor antagonists may not preclude clinical use for treating alcoholism during the day. In fact, the sleep promoting effects of an orexin-2 receptor antagonist at night could be an additional benefit to drug addicts, given that sleep disturbances are often associated with addiction, and treating such sleep disturbances have been shown to improve abstinence rates (Teran et al. 2008). Moreover, the occurrence of insomnia with alcoholism is a significant predictor of relapse to drinking (Brower et al. 2001) and sleep deprivation increases drug use and relapse (Hamidovic and de Wit 2009). Given that the majority of alcoholics also have insomnia and that alcoholics often self-medicate that insomnia with alcohol (Brower et al. 2001), insomnia itself can promote alcohol abuse in a viscous cycle. In addition, actual drug intake in the middle of the night is also high in drug addicts, and night time drug use is associated with greater dependence, reduced abstinence, and increased relapse (Bover et al. 2008; Scharf et al. 2008). Therefore, an orexin-2 receptor antagonist may provide a novel addiction treatment that targets both day time and night time drug use to promote abstinence, while treating the insomnia comorbidity that promotes drug use and relapse.

Consistent with the data on self-administration, mice pretreated with the orexin-2 receptor antagonist failed to develop an ethanol place preference, suggesting that orexin-2 receptor blockade may have attenuated ethanol reward. JNJ-10397049 prevented the formation of ethanol place preference, when given before the ethanol conditionings, and tended to attenuate the expression of an already-formed ethanol place preference, when given before a CPP test. This suggests that orexin-2 receptor blockade may have

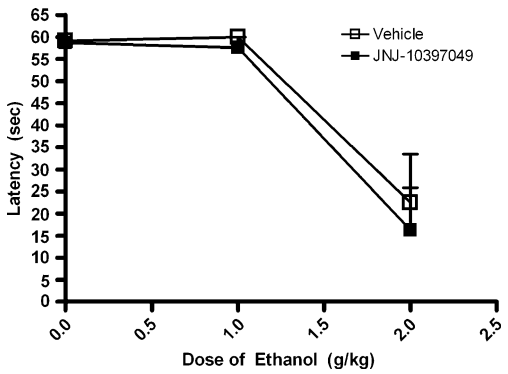

Fig. 7 Ethanol produced motor impairment as measured by rotarod performance ($p<0.05$). However, JNJ-10397049 produced no impairments by itself and did not affect the motor impairments produced by ethanol. N's are 8–16 per group

decreased ethanol intake by directly modulating the rewarding value of ethanol. The effects of orexin-2 receptor blockade on ethanol place preference were selective and not due to sedation since the compound did not modify locomotor activity by itself during the conditioning session. In addition, the effects of JNJ-10397049 were not due to any inherent aversive properties of the compound since JNJ-10397049 was void of any place conditioning properties when tested by itself. Due to the positive effects seen with the orexin-2 receptor antagonist on ethanol CPP and self-administration, we decided to further characterize it in a model of ethanol reinstatement. The orexin-2 receptor antagonist blocked ethanol-induced reinstatement in mice, suggesting that blockade of orexin-2 receptors may also promote alcohol abstinence clinically, by preventing lapses from turning into relapses.

We compared the effects of the orexin-2 receptor antagonist to naltrexone, a mu opioid receptor antagonist, one of the treatments currently used in the clinic for alcoholism. Naltrexone reduced ethanol self-administration, but also decreased saccharin self-administration and induced a conditioned place aversion (data not shown), consistent with clinical data indicating that it has aversive side-effects and a low compliance rate (Bouza et al. 2004; Hollister et al. 1981; Mark et al. 2003; Richards et al. 2008). Since JNJ-10397049 lacked these effects in rodents, it can be hypothesized that future drugs targeting the orexin-2 receptor might be without such aversive or dysphoric side-effects in humans, and thus might yield higher compliance rates and a better chance of successful treatment than naltrexone. Of course, blockade of orexin-2 receptors may produce a different subset of unwanted side-effects, since orexins are known to be involved in many processes and functions, such as arousal, food intake, etc.

We also wanted to evaluate whether orexin-2 receptor blockade would attenuate alcohol withdrawal signs in addition to the reinforcing effects. Four hours after ethanol discontinuation, animals displayed robust signs of withdrawal. Diazepam significantly and dose-dependently reduced ethanol withdrawal signs. However, neither JNJ-10397049 nor SB-408124 had an effect in this assay, suggesting that orexin-2 and -1 receptor antagonists may not reverse the stress and anxiety which are often observed in alcohol-dependent subjects.

In contrast to the orexin-2 receptor antagonist, SB-408124 did not modify the rewarding or reinforcing effects of ethanol in rats or mice. It is unlikely that the doses of SB-408124 used in our studies were not large enough to produce an effect. In fact, we have demonstrated that SB-408124 (30 mg/kg) fully occupies the orexin-1 receptor, has significant exposure in plasma and brain, and is biologically active in rats (Dugovic et al. 2009). The lack of effect of SB-408124 is in disagreement with three earlier reports, which demonstrated effects of SB-334867, another orexin-1 receptor antagonist, on ethanol intake, albeit with maximal effects at high doses (20–30 mg/kg; Lawrence et al. 2006; Moorman and Aston-Jones 2009; Richards et al. 2008). Interestingly, the study by Moorman showed that SB-334867 only had effects in rats with high ethanol intake and preference, and the study by Lawrence used rats selectively bred for high ethanol intake. Although Richards did not select rats for high ethanol intake or preference, in their study, control rats lever-pressed ~150 times for 0.1 ml of 10% ethanol on an FR3 schedule, while in our study, conducted in Wistar rats, control animals lever-pressed ~50 for 0.1 ml of 8% ethanol on the same schedule. Therefore, it is possible that activity at orexin-1 receptors is only recruited during very high levels of ethanol intake, or only involved during very high motivation to consume ethanol. Consistent with this, a recent paper showed that orexin-1 receptor activation was involved in high effort work for highly salient rewards (Borgland et al. 2009). However, future experiments would be needed to further examine this hypothesis.

Another possibility is that blockade of orexin-1 receptors may affect ethanol reinstatement to a larger extent than intake. For example, SB-334867 blocked reinstatement of food seeking without altering food self-administration (Richards et al. 2008; but see opposite effects in Nair et al. 2008) and blocked cocaine reinstatement without altering cocaine intake, while orexin-A reinstated cocaine seeking without altering cocaine intake (Boutrel et al. 2005; Richards et al. 2008; Smith et al. 2009; but see a regulation of cocaine intake in Borgland et al. 2009 and Espana et al. 2010). In addition, SB-334867 was more potent at decreasing ethanol reinstatement (maximal effect at 5 mg/kg) compared to ethanol self-administration (maximal effect at 20 mg/kg; Richards et al. 2008). Our study did not examine the effects of SB-408124 on ethanol reinstatement, and future experiments are needed to test this hypothesis.

It is also possible that SB-408124 was ineffective in our study unlike SB-334867, the orexin-1 receptor antagonist used in previous studies, due to differences between the two compounds. Several previous studies tested only a single high dose of SB-334867 (20 or 30 mg/kg i.p.) or reported maximum effects at high doses (20–30 mg/kg i.p.; Boutrel et al. 2005; Harris et al. 2005; Lawrence et al. 2006; Richards et al. 2008; Sakurai et al. 1998; Sharf et al. 2008). Brain levels of SB-334867 reach about 14 μM at only 10 mg/kg i.p., which should be more than enough for full effects in vivo, given its Ki of about 60 nM (Upton 2005). In fact, our data show near maximal orexin-1 receptor occupancy (85%) with brain levels of SB-334867 at only 3–5 μM (after dosing with 10 mg/kg s.c., unpublished results). Therefore, maximum effects in vivo of SB-334867

should be observed at doses lower than 20–30 mg/kg i.p., when the orexin-1 receptor is already fully saturated. Also, we and others (Nair et al. 2008) have observed a general disruptive effect in vivo with SB-334867 at 30 mg/kg s.c. or i.p. (as measured by EEG after 30 mg/kg s.c., our unpublished results, and abnormal behaviors after 30 mg/kg i.p. in Nair et al. 2008), while finding no abnormal EEG pattern or disruptive behavioral effects in vivo with SB-408124 up to 30 mg/kg s.c.. Therefore, when all of this is taken together, it is possible that the positive effects seen only at high doses with SB-334867 might be due to off-target activity. In fact, since SB-334867 has a Kb of about 1 μM for the orexin-2 receptor, and 30 mg/kg i.p. SB-334867 results in brain concentrations of about 25 μM (Upton 2005), it is even possible that the previously reported positive effects of SB-334867 on ethanol intake seen at high doses were due in part to orexin-2 receptor blockade. A recent review raises this alarm, stating "in vivo studies using high doses [of SB-334867] should be viewed cautiously because those doses may block both [orexin] receptors" (Scammell and Winrow 2011). However, further experiments using additional tool compounds or orexin-1 receptor knockout animals would be needed to support this hypothesis.

We then tested whether the orexin-2 receptor antagonist reduced the rewarding effects of ethanol by changing extracellular levels of DA in the brain, since the effects of orexin peptides on the reward system seem to be mediated by DA. For example, it has been shown that orexin peptides administered to the ventral tegmental area increase extracellular DA levels in the nucleus accumbens and produce a place preference, whereas prepro-orexin knockout diminishes drug-induced increases in extracellular levels of DA and drug-induced locomotor activation (Narita et al. 2006, 2007). Furthermore, orexin-1 antagonists have been shown to attenuate cocaine-induced increases in extracellular levels of DA (Espana et al. 2010) and the locomotor stimulating effects of orexins have been shown to be mediated by the dopaminergic system (Nakamura et al. 2000). In addition, JNJ-10397049 attenuated ethanol-induced hyperactivity, which is known to be mediated at least in part by increases in DA in the nucleus accumbens (Imperato and Di Chiara 1986; Meyer et al. 2009). Thus, it was surprising to see that ethanol-induced increases in DA levels in the nucleus accumbens were not altered by orexin-2 receptor blockade.

Another likely candidate involved in JNJ-10397049's effects on ethanol could be glutamate. For example, recent electrophysiological studies have demonstrated that the orexin-B peptide increases glutamatergic transmission and potentiates NMDAR current in the VTA (Borgland et al. 2008). Therefore, it is entirely possible that JNJ-10397049 attenuated the reinforcing effects of ethanol by modulating glutamate transmission. An alternative mechanism of action could be a direct modulation of nucleus accumbens activity by orexin-2 receptors, since orexin-B directly depolarizes neurons in the nucleus accumbens (Mukai et al. 2009), and the firing pattern of the nucleus accumbens is believed to encode goal-directed behavior and drug-reinforcement (Carelli 2002; Robinson and Carelli 2008). It will be very interesting to determine the mechanism of orexin-2 receptor blockade on ethanol intake, especially if it involves a novel, non-dopaminergic pathway.

In conclusion, the results of these studies demonstrate for the first time that selective blockade of orexin-2 receptors reduce the rewarding and reinforcing effects of ethanol without changing the responses for a palatable solution of saccharin and without producing aversive effects as measured by place aversion. Although the orexin-1 receptor antagonist was not effective in these same studies, that does not preclude an involvement of the orexin-1 receptor in ethanol seeking, especially perhaps during conditions of high motivation/work effort or during reinstatement, and certainly does not diminish the already established role of the orexin-1 receptor in psychostimulant and food reward. However, these studies do highlight the possibility that orexins mediate reward processes through the orexin-2 receptor, as well as the orexin-1 receptor, and warrant more research on the involvement of the orexin-2 receptor in addiction. Furthermore, these data indicate that orexin-2 receptor antagonists may be useful for the treatment of alcoholism, especially when associated with sleep disturbances.

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