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Involvement of the orexin/hypocretin system in ethanol conditioned place preference

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Abstract

Rationale—Recent studies suggest that orexin/hypocretin is involved in drug reward and drug-seeking behaviors, including ethanol self-administration. However, orexin's role in ethanol-induced seeking behaviors remains unclear.

Objective—These studies examined the role of orexin in the acquisition and expression of ethanol conditioned place preference (CPP) using the orexin 1 receptor (OX1R) antagonist SB-334867.

Methods—Effects of SB-334867 (0–30 mg/kg) on locomotor activity were determined in DBA/2J mice (Experiment 1). SB-334867 (0–30 mg/kg) was administered during acquisition of ethanol (2 g/kg) CPP to determine whether orexin signaling is required (Experiment 2). Blood ethanol concentrations (BECs) were measured after ethanol (2 g/kg) injection to determine whether SB-334867 (30 mg/kg) pretreatment altered ethanol pharmacokinetics (Experiment 3). Finally, SB-334867 (0–40 mg/kg) was given before ethanol-free preference testing (Experiments 4 and 5).

Results—SB-334867 did not alter basal locomotor activity (Experiment 1). SB-334867 (30 mg/kg) reduced ethanol-induced locomotor stimulation, but did not affect the acquisition of ethanol CPP (Experiment 2) or BEC, suggesting no alteration in ethanol pharmacokinetics (Experiment 3). Although OX1R antagonism blocked expression of a weak ethanol CPP (Experiment 4), it did not affect expression of a moderate to strong CPP (Experiment 5).

Conclusions—Blockade of OX1R by systemic administration of SB-334867 reduced ethanol-stimulated activity, but did not affect acquisition or expression of ethanol-induced CPP, suggesting that orexin does not influence ethanol's primary or conditioned rewarding effects. Other neurotransmitter systems may be sufficient to support acquisition and expression of CPP despite alterations in orexin signaling.

Keywords

Conditioned place preference; SB-334867; Orexin; Locomotor activity; Inbred mice (DBA/2J)

Introduction

The neuropeptides orexin A and B (also called hypocretin 1 and 2) were discovered in 1998 and shown to have behavioral effects on arousal and feeding (de Lecea et al. 1998; Hara et al. 2001; Sakurai et al. 1998). These peptides are expressed mainly in neurons of the perifornical region and lateral hypothalamus (LH), though orexin fibers and receptors are widely distributed throughout the brain (de Lecea et al. 1998; Peyron et al. 1998). This ubiquity is consistent with orexin's wide array of physiological functions (Peyron et al. 1998). There are two G-protein coupled orexin receptor subtypes, orexin 1 receptor (OX1R), which has higher affinity for orexin A, and orexin 2 receptor (OX2R), which has high affinity for both peptides (Sakurai et al. 1998). Though orexins have been well characterized for their role in arousal and feeding, a recent surge of research has provided increasing evidence for orexin involvement in drug reward and drug-seeking behaviors (Harris and Aston-Jones 2006; Aston-Jones et al. 2009, 2010; Bonci and Borgland 2009). Consistent with this function, orexin fibers project to regions important for reward processing such as the ventral tegmental area (VTA), amygdala, and nucleus accumbens (NAc; Fadel and Deutch 2002; Peyron et al. 1998). Of particular relevance to addiction, orexin is hypothesized to affect glutamate-mediated responses of VTA dopamine neurons (Aston-Jones et al. 2009, 2010).

Much of the initial evidence for this hypothesis came from studies using the conditioned place preference (CPP) procedure. For example, bilateral lesion of orexin neurons in the LH (Harris et al. 2007) or intra-VTA injection of the selective OX1R antagonist SB-334867 (Narita et al. 2006) interfered with acquisition of morphine-induced CPP in rats. Moreover, a disconnection technique showed that unilateral lesion of the LH and contralateral intra-VTA infusion of the OX1R antagonist blocked acquisition of morphine CPP, although neither treatment had any effect on its own, suggesting that the orexin projection from LH to VTA is essential for morphine reward or for learning to associate a stimulus context with morphine's effects (Harris et al. 2007). Studies have also linked orexin to expression of drug-conditioned behavior. For example, orexin neurons were activated in proportion to the magnitude of morphine CPP expressed and activation of OX1Rs in the VTA was sufficient to reinstate an extinguished morphine CPP (Harris et al. 2005). Blocking OX1Rs also disrupted expression of morphine CPP (Harris et al. 2005) and cue or context induced reinstatement of an extinguished lever press response previously reinforced by cocaine (Smith et al. 2009, 2010), suggesting that these receptors are important for the expression of drug-induced conditioned motivational or conditioned rewarding effects (i.e., drug seeking). However, OX1R blockade had no effect on established cocaine self-administration, indicating no role for orexin signaling in cocaine's primary reinforcing effect (Smith et al. 2009).

In contrast, relatively less is known about the role of orexin in ethanol-rewarded behaviors. One recent study showed that orexin-A injected into the LH or paraven-tricular nucleus (but not NAc) selectively induced ethanol intake rather than food intake in rats (Schneider et al. 2007). Another study showed a trend for a correlation between OX1R gene expression in the hypothalamus and ethanol self-administration in rats (Pickering et al. 2007). Further supporting a role for OX1R in ethanol consumption by rats, OX1R antagonism reduced ethanol intake in a two-bottle choice drinking procedure (Moorman and Aston-Jones 2009) and in operant self-administration procedures (Lawrence et al. 2006; Richards et al. 2008). OX1R blockade also interfered with reinstatement of an extinguished operant response induced by yohimbine (Richards et al. 2008) or a cue previously paired with access to ethanol (Lawrence et al. 2006). Moreover, an ethanol-paired stimulus that produced reinstatement was also found to activate hypothalamic orexin neurons (Dayas et al. 2008).

Thus, in contrast to cocaine (Smith et al. 2009, 2010), orexin signaling may be involved in both the primary as well as the conditioned reinforcing effects of ethanol.

Interpretation of drug pretreatment effects on oral ethanol self-administration is potentially complicated because oral ethanol intake is sometimes reinforced more by ethanol's taste and calories than by its presumed rewarding post-absorptive pharmacological effects (Cunningham et al. 2000; Dole et al. 1985; Leeman et al. 2010). Given orexin's prominent role in feeding behavior, it is important to also study its role in an ethanol reward model that minimizes the role of taste and is more clearly based on induction of a strong pharmacological effect. Thus, the present studies used the CPP procedure, which was previously used successfully to characterize orexin's role in morphine reward (Harris et al. 2005, 2007; Narita et al. 2006). More specifically, the present experiments were designed to determine whether orexin influences the acquisition (Experiment 2) or expression (Experiments 4 and 5) of ethanol-induced CPP using systemic administration of the selective OX1R antagonist SB-334867 in DBA/2J mice. SB-334867 has approximately 50-fold selectivity for the OX1R over the OX2R (Porter et al. 2001; Smart et al. 2001). SB-334867 easily penetrates the CNS, and in rats, peak plasma and brain concentrations are achieved at 30 min after intraperitoneal (IP) administration (Ishii et al. 2005). Given previous findings with morphine, cocaine, and ethanol in rats (see above), we expected acquisition and expression of ethanol CPP to be reduced or eliminated due to blockade of OX1R signaling in brain regions such as the VTA and amygdala, which have been shown to be important for expression of ethanol-induced CPP (Bechtholt and Cunningham 2005; Gremel and Cunningham 2009).

Materials and methods

Animals

Male DBA/2J mice arrived at 6–9 weeks of age from Jackson Laboratory (Davis, CA) and were housed in groups of four in polycarbonate cages with corncob bedding. The cages were housed in a ventilated Thoren rack. Mice were allowed to acclimate to the housing environment for 2 weeks before any experimental procedures. The colony room was kept at approximately 21°C on a normal 12 h light cycle (lights on 7 am–7 pm) and experiments were conducted during the day. Food and water were available continuously in the home cage. The Oregon Health and Science University Institutional Animal Care and Use Committee approved all experimental procedures, which followed the National Institutes of Health "Principles of Laboratory Animal Care."

Apparatus

Twelve conditioning boxes (30×15×15 cm) were enclosed in light and sound-attenuating chambers equipped with a fan (55.9×40.6×45.7 cm; Coulbourn Instruments, Whitehall, PA; Model E10-20). All experimental procedures were conducted without lights in these chambers. The long sides and lid of the conditioning boxes were made of acrylic and the end panels were made of aluminum. Photodetectors and infrared light sources were mounted 2.2 cm above the floor of the conditioning box at 5-cm intervals along the sides of the box. A computer recorded activity and position of mice within the conditioning box. Interchangeable grid and hole floor halves were used as tactile conditioned stimuli (CSs). During conditioning trials, matching floor halves were placed under the conditioning box (i.e., one compartment training procedure). On preference test days, one of each floor type was presented, counterbalanced for position within each group. The grid floor was constructed of 2.3 mm stainless steel rods attached 6.4 mm apart on acrylic rails. The hole floor was made of 16 gauge stainless steel with 6.4-mm diameter round holes on 9.5 mm staggered centers. Previous studies have shown that groups of saline-treated DBA/2J mice

show approximately equal preference for each tactile floor cue (Cunningham et al. 2003). A picture of the apparatus has been published (Cunningham et al. 2006).

Drugs

Ethanol (20%, v/v) was prepared from a 95% stock solution diluted in saline (0.9% NaCl) and injected IP at 2 g/kg (12.5 ml/kg). This ethanol dose has repeatedly been shown to rapidly condition a strong place preference in DBA/2J mice (e.g., Groblewski et al. 2008). SB-334867 (*N*-(2-Methyl-6-benzoxazolyl)-*N'*-1,5-naphthyridin-4-yl urea, Tocris Bioscience, Ellisville, MO) was prepared in a stock concentration of 1.5 mg/ml and suspended in 20% (w/v) hydroxypropyl- β -cyclodextrin and 1.5% (v/v) dimethyl sulfoxide (DMSO) in saline. Due to the low solubility of SB-334867, β -cyclodextrin has been widely used as the vehicle in many studies of the antagonist's effects on drug-related behaviors (e.g., Moorman and Aston-Jones 2009; Richards et al. 2008; Smith et al. 2009, 2010). SB-334867 or vehicle was injected IP at a volume of 20 ml/kg unless noted otherwise. SB-334867 was administered at doses of 0, 10, 15, 20, 30, or 40 mg/kg. Systemic doses in this range have been reported to be effective in both rats and mice in several different behavioral procedures (Harris et al. 2005; Lawrence et al. 2006; Moorman and Aston-Jones 2009; Richards et al. 2008; Rodgers et al. 2001; Sharf et al. 2008; Smith et al. 2009, 2010).

Experiment 1: SB-334867 effects on locomotor activity

Because the effects of SB-334867 on locomotor activity had not previously been studied in DBA/2J mice, Experiment 1 examined locomotor effects of SB-334867 (0, 10, 20, or 30 mg/kg) in a between-subjects design ($n = 12/\text{group}$). This experiment did not include the 40 mg/kg dose (used later only for the final test in Experiment 5) because the literature suggested it was beyond the dose range that was expected to be effective in altering CPP. Mice were first habituated to handling and injection in a single session 3 days before the activity test session. During this habituation session, saline was injected immediately before a 5-min exposure to the conditioning apparatus on a white paper floor. On the test day, mice were randomly assigned to groups and received their assigned dose of SB-334867 immediately before placement into the chamber for 60 min.

General behavioral procedures for CPP experiments (Experiments 2, 4, and 5)

Habituation—A one-day habituation procedure preceded conditioning in Experiments 2, 4, and 5. The purpose of this session was to adapt mice to handling and injection procedures. Animals were injected with saline (0.9% NaCl, 12.5 ml/kg) and placed in the conditioning apparatus on a white paper floor for 5 min.

Conditioning—An unbiased place-conditioning procedure was used (Cunningham et al. 2003). Mice were randomly assigned to two conditioning subgroups (G+ and G−). The G+ subgroup received an IP injection of ethanol (2 g/kg) immediately before exposure to the matching grid floor halves (CS+ trials) and saline before exposure to the hole floors (CS− trials). In contrast, the G− subgroup received ethanol paired with the hole floor and saline with the grid floor. Mice received only one trial per day and each trial lasted 5 min. Conditioning consisted of eight sessions (four CS+ trials and four CS− trials on alternating days); order of exposure to each type of trial was counterbalanced within subgroups. Pretreatment injections were given in the home cage 30 min before conditioning trials in Experiments 2 (vehicle or SB-334867) and 5 (vehicle only). This conditioning procedure has been repeatedly shown to induce a strong ethanol CPP in DBA/2J mice in our laboratory (Cunningham et al. 2006).

Preference test—Preference testing occurred after the second pair of conditioning trials and after the fourth pair of trials (Experiment 2 and 5) or after the fourth pair of trials only (Experiment 4). Testing mice after only two pairs of conditioning trials provided an opportunity to assess antagonist pretreatment effects at an intermediate point in training, thereby reducing concerns that pretreatment effects might be obscured by a response ceiling (Groblewski et al. 2008). All mice received a saline injection immediately before placement in the center of the conditioning apparatus with both floor types for each 30-min test. Pretreatment injections (vehicle or SB-334867) were given in the home cage 30 min before tests in Experiments 4 and 5. Test session activity data (mean counts/min) were recorded to aid in the interpretation of preference data (see Gremel and Cunningham 2007).

Experiment 2: SB-334867 effects on CPP acquisition

This experiment was designed to determine whether OX1R signaling is involved in the development of ethanol CPP. Conditioning proceeded as described above in “General behavioral procedures for CPP experiments.” The OX1R antagonist SB-334867 (0, 15, or 30 mg/kg) was administered 30 min before CS+ trials ($n=14-16$ per conditioning subgroup). All mice received vehicle injections 30 min before CS- trials. Mice were tested 48 h after the second pair of trials and 48 h after the fourth pair of trials in 30-min place preference tests.

Experiment 3: SB-334867 effects on ethanol pharmacokinetics

To address the possibility that SB-334867 altered ethanol absorption or distribution, Experiment 3 examined blood ethanol concentrations (BEC) in naive mice pretreated with vehicle or 30 mg/kg SB-334867 ($n=10$ /group) 30 min before injection of ethanol (2 g/kg). A third group pretreated with saline ($n=10$) was also included to address the possibility that the β -cyclodextrin vehicle affects BEC. We chose to test the 30 mg/kg SB-334867 dose because it was the only dose that produced a significant behavioral effect in Experiment 2 (see “Results”). After 1 week acclimation to the colony, blood samples (20 μ l/sample) were collected from the saphenous vein at 10 and 30 min after ethanol injection in a within-subjects design. Due to premature clotting and other problems, samples of sufficient volume could not be obtained from one or two mice in each group at each time point. Thus, the final group sizes were 8–9 per group. Samples were analyzed using gas chromatography (Rustay and Crabbe 2004).

Experiment 4: SB-334867 effects on CPP expression (no vehicle habituation)

This experiment was designed to determine if acute OX1R activation is required for expression of ethanol CPP. Conditioning proceeded as described above in “General behavioral procedures for CPP experiments.” Twenty-four hours after the fourth pair of trials, the OX1R antagonist SB-334867 (0, 15, or 30 mg/kg) was injected 30 min before the place preference test. An initial group of 96 mice was used for this experiment ($n=14-16$ per conditioning subgroup). However, the vehicle (0 mg/kg) control group showed a CPP that was much weaker than expected (see “Results”) when compared to other recent studies that used identical conditioning parameters, but no pretest treatment injection (e.g., Groblewski et al. 2008). Thus, the experiment was repeated in a second replication of 96 mice to determine whether the behavior of the 0 mg/kg control group was due to sampling error.

Experiment 5: SB-334867 effects on CPP expression (after vehicle habituation)

Given the apparent disruption of CPP expression in the vehicle (0 mg/kg) control group in Experiment 4, Experiment 5 was performed to further investigate CPP expression using a procedure intended to habituate mice to the pretreatment vehicle injection before testing. Conditioning proceeded as described above in “General behavioral procedures for CPP

experiments,” except that 30 min before every conditioning trial (CS+ and CS–), all mice received a vehicle pretreatment injection in the home cage. Preference tests occurred 24 h after the second pair of trials and 24 h after the fourth pair of trials. The OX1R antagonist SB-334867 (0 or 30 mg/kg) was injected 30 min before the test after two trials. A third group (No Pretreat) received only the saline injection immediately before the test with no pretreatment injection in the home cage. The purpose of this additional control group was to provide a “normal CPP” baseline against which to compare the 0 mg/kg pretreatment group. If the habituation procedure successfully eliminated whatever had disrupted CPP in Experiment 4, the No Pretreat and 0 mg/kg groups would be expected to express similar levels of CPP. Because 30 mg/kg had no effect on CPP after 2 trials, the antagonist dose was increased to 40 mg/kg (by increasing injection volume) and was injected 30 min before the final test to the SB-334867 group and to the No Pretreat group (previously SB-334867 naive). All conditioning subgroups contained 15–16 mice.

Statistical analyses

The primary dependent variable on CPP expression tests was time spent on the grid floor. Seconds spent on the grid side was divided by total duration of the test session in min (i.e., 30), creating a dependent variable indexed in s/min. This simple transformation allows ready comparison to the full range of possible outcomes (e.g., 0 s/min=complete aversion to grid; 60 s/min = complete preference for grid). Also, because there are only two possible locations, time spent on the hole side is reflected in the difference between mean time on grid and the 60 s/min maximum. The difference in time spent on the grid floor between mice that had ethanol paired with the grid floor (G+) and mice that had saline paired with the grid floor (G–) indexes CPP strength (Cunningham et al. 2003). Preference data were initially analyzed using a three-way (Group×Conditioning Subgroup×Time) analysis of variance (ANOVA). For all experiments, “group” refers to the antagonist pretreatment groups, “conditioning subgroup” refers to G+/G– assignment, and “time” refers to the first 15 min versus last 15 min of the test. Mean time on the grid floor during the first and second half of the preference test was compared to determine if there were time-dependent changes in the strength of CPP expression. When multiple preference tests were conducted (Experiment 2 and 5), a mixed ANOVA (Group×Conditioning Subgroup×Test) was also used to analyze data across tests. Replication was included as an additional between-subjects factor in analyses of Experiment 4.

Test session activity data were analyzed using a mixed ANOVA (Group×Time) or a one-way ANOVA (Group). Conditioning trial activity data were analyzed using a mixed ANOVA (Group×Trial×Trial type). “Trial type” refers to whether saline (CS–) or ethanol (CS+) was administered before the trial. In Experiment 2, conditioning activity data were also analyzed using a mixed ANOVA that included minutes as an additional within-subjects factor.

BEC data were analyzed using one-way ANOVAs at each time point. The α -level for all analyses was $p=0.05$. All post hoc analyses were Bonferroni-corrected pairwise comparisons.

Results

Subject attrition

In Experiment 2, two mice were excluded because of a procedural error, one mouse died, and one mouse was excluded because of an injection injury making the total sample size 92. In Experiment 4, five mice were removed for procedural errors making the total sample size

187. In Experiment 5, two mice died, two mice were removed due to a procedural error, and one mouse was removed for an injection injury making the total sample size 91.

Experiment 1: SB-334867 effects on locomotor activity

Activity counts for each of the four dose groups (0, 10, 20, and 30 mg/kg) are plotted in 5 min time bins over the 60-min session in Fig. 1. Although activity decreased over time (presumably due to habituation), there was no acute effect of SB-334867 on activity. A Group $\times$ Time ANOVA supported these conclusions, yielding a significant main effect of time [$F(11,484)=39.9, p<0.0001$], but no effect of dose or interaction. Thus, this study suggested that SB-334867 should not alter locomotor activity when DBA/2J mice are tested within 60 min after antagonist treatment in a CPP procedure.

Experiment 2: SB-334867 effects on CPP acquisition

Preference tests—Mean times spent on the grid floor by each dose group during the preference tests after two pairs of conditioning trials (left panel) and after 4 pairs of conditioning trials (right panel) are shown in Fig. 2. On both tests, G+ subgroups spent more time on the grid floor than the G− subgroups, indicating development of a place preference. As expected, CPP magnitude was stronger after four conditioning trials than after two conditioning trials. However, there was no effect of SB-334867 during either test.

These observations were supported by two-way (Group $\times$ Conditioning Subgroup) ANOVAs conducted separately for each test, which yielded significant main effects of conditioning subgroup [F values(1,86) $\geq 100.2, p$ values < 0.001], but no group effect or interaction, indicating that all groups showed a significant CPP of similar magnitude on both tests. To address time-dependent changes in CPP magnitude within each test, separate three-way ANOVAs (Group $\times$ Conditioning Subgroup $\times$ Time) were also performed. These analyses yielded a significant conditioning subgroup $\times$ time interaction on test 2 [$F(1,86)=7.4, p<0.008$], and a marginal conditioning subgroup $\times$ time interaction on test 1 [$F(1,86)=3.4, p<0.07$], reflecting stronger preference during the first half of each test (data not shown). To address the effect of number of conditioning trials, a three-way (Group $\times$ Conditioning Subgroup $\times$ Test) ANOVA was conducted. Preference was significantly larger after four conditioning trials as indicated by a significant conditioning subgroup $\times$ test interaction [$F(1,86)=90.2, p<0.001$]. There were no group differences in locomotor activity on either test (Table 1).

Conditioning activity—Consistent with previous studies in DBA/2J mice, activity on ethanol trials exceeded that on saline trials, confirming the locomotor-stimulating effect of the 2 g/kg dose. Unexpectedly, pretreatment with SB-334867 dose-dependently reduced the increase in locomotor activity normally produced by ethanol. Mean activity counts per min ($\pm$ SEM) on ethanol trials were 161.9 (± 5.0), 149.6 (± 4.3), and 137.1 (± 4.0) for the 0, 15, and 30 mg groups, respectively. On saline trials (after pretreatment with vehicle), group means were 57.5 (± 1.8), 60.7 (± 1.6), and 59.6 (± 1.8). Three-way ANOVA (Group $\times$ Trial $\times$ Trial Type) indicated that all main effects were significant (p values < 0.02) as well as the trial $\times$ trial type [$F(3,267)=30.0, p<0.0001$] and group $\times$ trial type [$F(2,89)=11.5, p<0.0001$] interactions. The trial $\times$ trial type interaction reflected a general divergence over trials between activity on CS+ versus CS− trials, due both to sensitization across ethanol trials and habituation across saline trials (data not shown). Follow-up tests indicated that the group $\times$ trial type interaction was explained by a simple main effect of group on ethanol trials [$F(2,89)=7.7, p=0.001$], but not on saline trials [$F<1$]. Average ethanol activity for the 30 mg/kg group was significantly lower than for the 0 mg/kg group (Bonferroni corrected $p<0.001$). The 15 mg/kg group did not differ significantly from either of the other groups.

To further examine the effect of the OX1R antagonist on ethanol-stimulated activity, mean activity counts were examined during each minute of the conditioning sessions, averaged over trials. As can be seen in Fig. 3, SB-334867 did not affect activity during the first 2 min of ethanol trials. However, the antagonist dose-dependently reduced ethanol-stimulated activity during the last 3 min, indicating rapid development of the effect within a few minutes after injection. Three-way (Group×Trial Type×Minute) ANOVA yielded significant outcomes for all main effects and interactions (p values<0.02), including the three-way interaction [$F(8,356)=5.8, p<0.0001$], which reflected a significant group×minute interaction on ethanol trials [$F(8,356)=8.2, p<0.0001$], but not on saline trials.

Summary—SB-334867 reduced locomotor activation to ethanol at the high dose (30 mg/kg), but antagonist pretreatment did not affect acquisition of ethanol-induced CPP. Thus, these findings suggest that blockade of OX1R does not alter the ability of mice to associate environmental stimuli with ethanol's rewarding effect.

Experiment 3: SB-334867 effects on ethanol pharmacokinetics

Mean BECs (mg/ml ± SEM) are shown for each group in Table 2. There was no difference between the 0- (β-cyclodextrin vehicle) and 30 mg/kg groups at either time point. Although BEC in the saline pretreated group was slightly higher at 10 min, all groups showed similar BECs 30 min after ethanol injection. One-way ANOVAs confirmed the absence of a group effect at 30 min ($F<1$), but revealed a significant group effect at 10 min [$F(2,22)=3.8, p<0.05$], reflecting higher BEC after pretreatment with saline than after pretreatment with 30 mg/kg SB-334867 (Bonferroni-adjusted $p=0.04$). However, there was no significant difference between the saline and 0 mg/kg groups, indicating that the β-cyclodextrin vehicle did not affect ethanol pharmacokinetics. Overall, these data indicate that ethanol absorption and distribution were not altered by SB-334867.

Experiment 4: SB-334867 effects on CPP expression (no vehicle habituation)

Preference test—Figure 4 shows mean times spent on the grid floor by each group during the first 15 min of the preference test collapsed across both replications of the study. Unexpectedly, CPP was reduced compared to Experiment 2. Nevertheless, the higher antagonist dose (30 mg/kg) appeared to interfere with CPP expression. Two-way ANOVA (Group × Conditioning Subgroup) confirmed overall expression of a significant preference [significant main effect of conditioning subgroup, $F(1,181)=33.3, p<0.0001$]. However, the group×conditioning subgroup inter-action fell short of the criterion for significance [$F(2,181)=2.1, p=0.12$]. Post hoc comparisons between the G+ and G− conditioning subgroups within each pretreatment drug condition showed significant CPP in the 0 and 15 mg/kg groups (Bonferroni-corrected p values<0.001), but not in the 30 mg/kg group, suggesting blockade of CPP expression at the higher dose.

The reduced CPP in the vehicle (0 mg/kg) group (relative to previously published studies) did not appear to be due to sampling error because the effect was present in both replications (i.e., a Group×Conditioning Subgroup×Replication ANOVA yielded no significant interactions between replication and either of the other factors). Also, in contrast to Experiment 2, CPP was not maintained throughout the entire 30-min test. Means (±SEM) during the last 15 min were 27.3 (±1.6) and 30.6 (±1.9) for the G+ and G− groups, respectively. A three-way ANOVA that included time as a factor (Group×Conditioning Subgroup×Time) supported this observation by showing a significant conditioning subgroup×time interaction [$F(1,181)=74.1, p<0.0001$], reflecting the significant main effect of conditioning subgroup during the first 15 min (see above), but not during the last 15 min [$F(1,181)=1.9, p=0.18$]. There were no group differences in activity during the first 15 min of testing (Table 1).

Conditioning activity—Overall mean activity rates ($\pm$ SEM) were 144.1 ($\pm$ 2.5) and 55.1 ($\pm$ 0.9) counts/min on CS+ and CS− trials, respectively. Three-way ANOVA (Group $\times$ Trial $\times$ Trial Type) yielded the expected main effect of trial type [$F(1,184)=1555.2$, $p<0.0001$] and a trial $\times$ trial type interaction [$F(3, 552)=10.2$, $p<0.0001$], reflecting divergence between CS+ and CS− activity across days (data not shown).

Summary—The high dose of SB-334867 (30 mg/kg) blocked expression of ethanol CPP. However, this finding was complicated by an unexpectedly low preference in the vehicle control group, an effect that may have been caused by the novelty of receiving a pretreatment injection or novelty of the vehicle solution. Thus, Experiment 5 was designed to further examine OX1R antagonist effects on CPP expression by using habituation to vehicle injection and pretreatment handling to reduce or eliminate these potential influences on test performance.

Experiment 5: SB-334867 effects on CPP expression (after vehicle habituation)

Preference tests—Figure 5 shows mean time spent on the grid floor by each group on the tests after the first two pairs of conditioning trials (left panel) and after all four pairs of trials (right panel). On both tests, all groups expressed a significant preference of similar magnitude, indicating no effect of the OX1R antagonist, even after the dose was increased to 40 mg/kg on the second test. Moreover, the Vehicle (0 mg/kg) Pretreatment group and the No-Pretreat group displayed similar preferences on the first test, suggesting that the pretreatment habituation procedure eliminated any disruption in performance that might be related to novelty of the vehicle pretreatment injection. On the test after four conditioning trials, both of the groups that received 40 mg/kg SB-334867 showed preferences similar to the vehicle (0 mg/kg) control group, indicating no effect of the drug on expression of CPP.

Two-factor (Group $\times$ Conditioning Subgroup) ANOVAs conducted separately for each test yielded significant main effects of conditioning subgroup [F values (1, 85) >46.3 , p values <0.0001], but no main effect of group or interaction, indicating that SB-334867 did not alter CPP expression in either test. As in the case of Experiment 2, separate three-way ANOVAs (Group $\times$ Conditioning Subgroup $\times$ Time), conducted to address possible time-dependent changes in CPP within each test, yielded a significant conditioning subgroup $\times$ time interaction on both tests [F values (1, 85) >13.9 , p values <0.001], reflecting a stronger preference during the first 15 min of each test (data not shown). Three-way (Group $\times$ Conditioning Subgroup $\times$ Test) ANOVA suggested there was a small increase in CPP magnitude after four pairs of conditioning trials compared to the test after 2 trials as shown by a marginal conditioning subgroup $\times$ test interaction [$F(1,85)=3.4$, $0.05<p<0.07$]. There were no group differences in activity on the second test, but there was a significant group effect during the first test [$F(2,88)=10.2$, $p<0.0001$], reflecting less activity in the 30 mg/kg group compared to the No-Pretreat group; the 0 and 30 mg/kg groups did not differ (Table 1).

Conditioning activity—Mean activity rates ($\pm$ SEM) were 171.6 ($\pm$ 3.2) and 58.2 ($\pm$ 1.0) counts/min on CS+ and CS− trials, respectively. Three-way ANOVA (Group $\times$ Trial $\times$ Trial Type) yielded the expected significant main effect of trial [$F(3,264)=12.1$, $p<0.0001$], trial type [$F(1,88)=1602.9$, $p<0.0001$] and a trial $\times$ trial type interaction [$F(3, 264)=23.9$, $p<0.0001$].

Summary—The results of Experiment 5 indicate that OX1R antagonism during expression testing does not affect expression of CPP when preference is strong and not subjected to disruption as in Experiment 4.

Discussion

These experiments, which are the first to investigate orexin signaling and ethanol-induced CPP, indicate that OX1R plays little or no role in the acquisition (Experiment 2) or expression (Experiment 5) of this form of ethanol-conditioned behavior. OX1R blockade failed to alter CPP whether tested at an intermediate level of performance (i.e., after two pairs of conditioning trials) or at a higher level of performance (i.e., after four pairs of conditioning trials), reducing concern that the lack of effect was due to insensitivity at the extremes of the performance range (Grolewski et al. 2008). Although the antagonist appeared to block the expression of a weak to moderate CPP (Experiment 4), this effect was most likely due to some interaction between the drug and novelty of the vehicle pretreatment procedure because the effect was absent in mice that were well habituated to vehicle pretreatment before testing (Experiment 5).

The inability of SB-334867 to reliably alter either the acquisition or expression of ethanol-induced CPP cannot be explained by insensitivity of our procedures. This laboratory has previously published many studies showing effects of various pharmacological or neurobiological manipulations on the acquisition (Boyce-Rustay and Cunningham 2004; Chester and Cunningham 1999b; Cunningham and Gremel 2006; Gremel and Cunningham 2008), expression (Bechtholt and Cunningham 2005; Gremel and Cunningham 2007, 2008, 2009, 2010), and extinction (e.g., Cunningham et al. 1995, 1998) of ethanol-induced CPP using the same mouse strain, equipment, and procedures described here. Thus, the place-conditioning procedures used here were appropriate for detecting potential effects of SB-334867.

SB-334867 dose and route of administration

Two potential issues in the interpretation of our studies are: (a) the selection of SB-334867 doses, and (b) the systemic route of administration. Given the significant effect of SB-334867 (30 mg/kg) on ethanol-stimulated activity (Experiment 2) and the use of doses that have produced significant behavioral effects in previous studies with both rats and mice, the absence of an effect on CPP is not easily attributed to inappropriate dose selection. Nevertheless, it is possible that systemic administration of SB-334867 at these doses did not produce the brain concentrations required in mice to block OX1R signaling and produce the hypothesized alterations in glutamate or dopamine transmission. Although a previous study reported reduction in morphine-induced CPP in rats using systemic administration of 30 mg/kg (Harris et al. 2005), more compelling evidence that the antagonist interfered with CPP was provided in later studies that involved infusion of SB-334867 into VTA (e.g., Harris et al. 2007; Narita et al. 2006). Future studies of ethanol-induced CPP in mice could address this issue by infusing SB-334867 directly into VTA or other brain regions to allow for more specific conclusions.

SB-334867 effects on ethanol stimulated activity

SB-334867 (0–30 mg/kg) did not affect basal locomotor activity (Experiment 1), a result consistent with previous studies using lower doses (0–20 mg/kg) in rats (Richards et al. 2008) and mice (Sharf et al. 2010), but inconsistent with a recent study showing that a 30 mg/kg dose reduced horizontal activity in rats (Smith et al. 2009). Although our studies showed no antagonist effect on basal activity, SB-334867 (30 mg/kg) significantly reduced locomotor stimulation to ethanol without altering BEC (Experiment 3) or acquisition of ethanol CPP (Experiment 2). The latter finding is in general agreement with previous studies from our laboratory that have shown dissociation between ethanol's rewarding and locomotor-stimulating effects (e.g., Chester and Cunningham 1999a; Risinger et al. 1992).

The antagonist's effect on ethanol-stimulated activity might indicate that orexin signaling is normally involved in modulating ethanol's low-dose stimulant effect or high-dose depressant effects. The presence of OX1Rs in the amygdala and VTA (Marcus et al. 2001) supports this hypothesis because both brain areas have been implicated in the control of ethanol-induced activation (Boehm et al. 2002; Gremel and Cunningham 2008). For example, SB-334867 might interfere with the normal modulating effect of endogenous orexin on dopamine neurons in VTA, thereby reducing ethanol-induced dopamine release, leading to a decrease in ethanol's stimulant effects.

Orexin's role in the primary and conditioned rewarding effects of ethanol

Previous studies have shown that SB-334867 reduced operant oral self-administration (Lawrence et al. 2006) and drinking (Moorman and Aston-Jones 2009) of ethanol in rats, but did not affect responding for water (Lawrence et al. 2006; Moorman and Aston-Jones 2009) or sucrose (Richards et al. 2008). Moreover, OX1R blockade interfered with reinstatement of an extinguished operant response induced by a cue previously paired with access to ethanol (Lawrence et al. 2006) or by exposure to a stressor (yohimbine; Richards et al. 2008). Such findings have been interpreted as evidence that the antagonist selectively reduced ethanol reward (Aston-Jones et al. 2009, 2010) or motivation to seek ethanol (Borgland et al. 2009).

In contrast, the present studies showed that OX1R played no role in the primary or conditioned rewarding effects of ethanol as indexed by CPP in mice. More specifically, the absence of an antagonist effect on CPP acquisition (Experiment 2) is not consistent with the suggestion that SB-334867 reduced ethanol's primary rewarding effect or the learning of a cue-ethanol association. Also, the absence of an antagonist effect on CPP expression (Experiment 5) is not consistent with the suggestion that SB-334867 interfered with ethanol's conditioned rewarding effect or retrieval of the cue-ethanol association. Lawrence et al. (2006) attributed the antagonist's effect on cue-induced reinstatement to blockade of a conditioned increase in endogenous orexin that normally activates neural pathways involved in ethanol seeking. Although one might expect similar processes to be engaged by exposure to an ethanol-paired floor cue during a CPP test, the absence of an antagonist effect in our studies fails to support this prediction.

One possible explanation for the discrepancy between the CPP studies and the oral self-administration studies might be that mice and rats differ either in the role that OX1R plays in ethanol seeking or in the efficacy of SB-334867. Since our studies are the first to examine effects of this antagonist on ethanol seeking in mice, this possibility is difficult to evaluate. However, other recent studies have shown significant effects of SB-334867 on morphine-seeking (Sharf et al. 2010) and nicotine-seeking (Plaza-Zabala et al. 2010) behavior in mice, indicating that OX1R's hypothesized broader role in drug seeking is not limited to rats. Nevertheless, it remains possible that OX1R is involved in ethanol seeking by rats, but is not involved in ethanol seeking by mice.

There are also several procedural differences between the rat and mouse ethanol studies that might underlie differences in the effect of SB-334867. For example, in all of the self-administration studies, rats received substantial cumulative exposure to ethanol over many days of acquisition training before antagonist testing, whereas mice in the CPP studies received relatively little (Experiments 4–5) or no (Experiment 2) exposure to ethanol before antagonist treatment, raising the possibility that SB-334867 reduced self-administration in rats by disrupting ethanol tolerance or sensitization. Other differences between the self-administration and CPP studies include: route of administration (oral vs. IP), ethanol dose per session (variable doses < 2.0 vs. 2.0 g/kg), rate of ethanol exposure (slow ingestion vs. rapid bolus injection), and the presence (self-administration) or absence (CPP) of ethanol's

olfactory cues. Any one or several of these differences might have affected activation of the orexin system or the efficacy of SB-334867. Another more general possibility is that OX1R plays a more important role in an instrumental learning procedure in which the animal controls drug exposure (e.g., self-administration) than in a Pavlovian conditioning procedure in which the experimenter controls drug exposure (e.g., CPP). However, this difference would presumably be unique to ethanol because the literature already includes several examples of SB-334867's influence on Pavlovian conditioning induced by experimenter administration of other drugs, including several CPP studies (e.g., Harris et al. 2005, 2007; Narita et al. 2006; Sharf et al. 2010).

Another potential explanation for the discrepancy is that the antagonist's effect in oral ethanol self-administration procedures might be related more to ethanol's taste and high caloric value (7 kcal/g) than to ethanol's presumed rewarding postabsorptive pharmacological effects. Although Richards et al. (2008) reported no effect of SB-334867 (20 mg/kg) on operant responding or reinstatement of responding for sucrose, more recent studies have shown that a lower SB dose will reduce breakpoint on a progressive ratio schedule involving a highly salient (high fat) food reward (Borgland et al. 2009) and that a higher SB dose (30 mg/kg) will reduce fixed-ratio responding for sucrose pellets in rats that are food-restricted or fed ad libitum (Cason et al. 2010). Moreover, the higher SB dose has also been reported to reduce cue-induced reinstatement of sucrose seeking in food-restricted rats (Cason et al. 2010). While not conclusive, such findings certainly leave open the possibility that SB-334867 reduced ethanol self-administration and reinstatement of ethanol seeking by interfering with orexin signaling related to the processing of ethanol as a high-calorie food.

Whether previously reported effects of SB-334867 on oral ethanol self-administration also reflected a reduction in ethanol's rewarding postabsorptive pharmacological effects is not clear. The ability of orally consumed ethanol to induce an intoxicating BEC depends critically on several factors including the rate at which ethanol is consumed and the contents of the stomach. For example, slow ingestion of a relatively large dose of ethanol with food in the stomach may produce little or no increase in BEC compared to the same dose given rapidly by IP injection (Goldstein 1983), even though the caloric content of both ethanol exposures is identical. Because none of the previous studies of OX1R antagonist effects on oral ethanol self-administration reported BECs achieved by vehicle control animals, the contribution of postabsorptive pharmacological effects to the behavioral results is unclear. Although it did not eliminate ethanol's calories, the rapid IP injection used in our studies produced a substantial BEC (Table 2), increasing confidence that mice experienced significant postabsorptive rewarding pharmacological effects. Thus, the discrepancy between the effects of SB on oral ethanol intake and CPP might reflect a difference between these two procedures in the relative contributions of ethanol's taste/calories and rewarding postabsorptive pharmacological effects to behavior.

In the case of oral self-administration, where ethanol's taste and calories sometimes play a greater role than ethanol's rewarding pharmacological effects (Leeman et al. 2010), SB might have reduced responding primarily via actions on feeding-related systems stimulated by ethanol's taste/calories or by CSs that engage those systems. For CPP, however, the ability to detect effects of SB on feeding-related systems activated by ethanol's taste/calories or ethanol-paired cues might have been obscured by SB's inability to offset stronger rewarding pharmacological effects of ethanol or conditioned reward based on such effects. Direct evidence for this hypothesized task difference in the balance between ethanol's caloric and pharmacological effects is lacking, but this issue should be addressed in future research that compares these two prominent models of ethanol reward. Finally, it is important to note that discordance between findings from CPP and self-administration

studies is not unique to ethanol, but has been noted for other abused drugs as well (Bardo and Bevins 2000).

Orexin's role in drug-reward and drug-seeking behavior

As noted earlier, the literature offers substantial evidence for a role of the orexin system in behaviors that reflect rewarding or incentive motivational effects of many abused drugs, including morphine (Harris et al. 2005, 2007; Narita et al. 2006; Sharf et al. 2010), cocaine (Boutrel et al. 2005; Smith et al. 2009, 2010), nicotine (Hollander et al. 2008; Plaza-Zabala et al. 2010), and ethanol (Dayas et al. 2008; Lawrence et al. 2006; Moorman and Aston-Jones 2009; Richards et al. 2008). However, studies involving SB-334867 have yielded a relatively complicated pattern of results that vary depending upon the target drug, behavioral procedure, and perhaps species. For example, antagonist pretreatment reduced nicotine (Hollander et al. 2008) and ethanol self-administration (Lawrence et al. 2006; Moorman and Aston-Jones 2009; Richards et al. 2008), but did not interfere with cocaine self-administration in rats (Smith et al. 2009). Furthermore, although SB-334867 interfered with the acquisition of stimulus-morphine learning (Harris et al. 2007; Narita et al. 2006), it had no effect on the acquisition of stimulus-cocaine (Smith et al. 2009) or stimulus-ethanol (Experiment 2) learning. Such findings indicate that OX1R's role in primary drug reward or in the learning of response-drug or stimulus-drug associations varies depending on the drug.

Evidence for OX1R's role in the processes that underlie reinstatement of an extinguished drug-seeking response has been more consistent across drugs. More specifically, these studies showed that SB-334867 interfered with reinstatement of responding for morphine (Harris et al. 2005), nicotine (Plaza-Zabala et al. 2010), cocaine (Boutrel et al. 2005; Smith et al. 2009, 2010), and ethanol (Lawrence et al. 2006; Richards et al. 2008). However, there have been differences across drugs in the antagonist's ability to interfere with the expression of CPP. Although systemic administration of SB-334867 reduced the expression of morphine-induced CPP in rats (Harris et al. 2005), it had no effect on expression of either cocaine-induced (Sharf et al. 2010) or ethanol-induced (Experiment 5) CPP in mice. Overall, this pattern of findings suggests that OX1R's role in the incentive motivational processes that underlie reinstatement and expression of CPP differs, depending on the drug used to establish drug-seeking behavior.

Aston-Jones et al. (2009, 2010) have proposed that orexin signaling may be critical only for events that depend on orexin's ability to potentiate glutamate-mediated responses of VTA dopamine neurons, thereby reconciling several of the differences across drugs and behavioral procedures. The suggestion that orexin might influence ethanol reward via actions in VTA is well supported by many findings, including recent data showing that low concentrations of ethanol increase glutamate release in VTA (Deng et al. 2009; Xiao et al. 2009). However, although previous research has implicated VTA opioid and GABA_B receptors in the expression of ethanol-induced CPP (Bechtholt and Cunningham 2005), the specific role of VTA glutamate responses during the acquisition or expression of ethanol-induced CPP remains unknown. One potential interpretation of the present findings is that SB-334867 failed to alter ethanol-induced CPP because VTA glutamate transmission plays a negligible role in the acquisition and expression of this form of ethanol seeking behavior. This issue should be addressed in future studies of the VTA's role in ethanol-induced CPP.

Summary and conclusions

In summary, the present studies show that OX1R signaling is not required for acquisition or expression of the conditioned rewarding effects of ethanol as indexed by the CPP procedure in mice. Nevertheless, OX1R blockade dose-dependently reduced ethanol-stimulated locomotor activity, an effect that cannot be explained by a change in ethanol

pharmacokinetics. The present studies do not diminish the substantial literature supporting the orexin system's role in the rewarding and incentive motivational effects of many abused drugs, but they clearly show that additional research is needed to more fully characterize the role of the orexin system in ethanol reward-related behaviors.

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References

- Aston-Jones G, Smith RJ, Moorman DE, Richardson KA. Role of lateral hypothalamic orexin neurons in reward processing and addiction. *Neuropharmacology*. 2009; 56(Suppl 1):112–121. [PubMed: 18655797]
- Aston-Jones G, Smith RJ, Sartor GC, Moorman DE, Massi L, Tahsili-Fahadan P, Richardson KA. Lateral hypothalamic orexin/hypocretin neurons: a role in reward-seeking and addiction. *Brain Res*. 2010; 1314:74–90. [PubMed: 19815001]
- Bardo MT, Bevins RA. Conditioned place preference: what does it add to our preclinical understanding of drug reward? *Psychopharmacology*. 2000; 153:31–43. [PubMed: 11255927]
- Bechtholt AJ, Cunningham CL. Ethanol-induced conditioned place preference is expressed through a ventral tegmental area dependent mechanism. *Behav Neurosci*. 2005; 119:213–223. [PubMed: 15727526]
- Boehm SL II, Piercy MM, Bergstrom HC, Phillips TJ. Ventral tegmental area region governs GABA(B) receptor modulation of ethanol-stimulated activity in mice. *Neuroscience*. 2002; 115:185–200. [PubMed: 12401333]
- Bonci A, Borgland S. Role of orexin/hypocretin and CRF in the formation of drug-dependent synaptic plasticity in the mesolimbic system. *Neuropharmacology*. 2009; 56(Suppl 1):107–111. [PubMed: 18694770]
- Borgland SL, Chang SJ, Bowers MS, Thompson JL, Vittoz N, Floresco SB, Chou J, Chen BT, Bonci A. Orexin A/hypocretin-1 selectively promotes motivation for positive reinforcers. *J Neurosci*. 2009; 29:11215–11225. [PubMed: 19741128]
- Boutrel B, Kenny PJ, Specio SE, Martin-Fardon R, Markou A, Koob GF, de Lecea L. Role for hypocretin in mediating stress-induced reinstatement of cocaine-seeking behavior. *Proc Natl Acad Sci USA*. 2005; 102:19168–19173. [PubMed: 16357203]
- Boyce-Rustay JM, Cunningham CL. The role of NMDA receptor binding sites in ethanol place conditioning. *Behav Neurosci*. 2004; 118:822–834. [PubMed: 15301608]
- Cason AM, Smith RJ, Tahsili-Fahadan P, Moorman DE, Sartor GC, Aston-Jones G. Role of orexin/hypocretin in reward-seeking and addiction: implications for obesity. *Physiol Behav*. 2010
- Chester JA, Cunningham CL. Baclofen alters ethanol-stimulated activity but not conditioned place preference or taste aversion in mice. *Pharmacol Biochem Behav*. 1999a; 63:325–331. [PubMed: 10371663]
- Chester JA, Cunningham CL. GABA(A) receptors modulate ethanol-induced conditioned place preference and taste aversion in mice. *Psychopharmacology (Berl)*. 1999b; 144:363–372. [PubMed: 10435409]
- Cunningham CL, Gremel CM. Proximal ethanol pretreatment interferes with acquisition of ethanol-induced conditioned place preference. *Pharmacol Biochem Behav*. 2006; 85:612–619. [PubMed: 17141849]
- Cunningham CL, Dickinson SD, Okorn DM. Naloxone facilitates extinction but does not affect acquisition or expression of ethanol-induced conditioned place preference. *Exp Clin Psychopharmacol*. 1995; 3:330–343.

- Cunningham CL, Henderson CM, Bormann NM. Extinction of ethanol-induced conditioned place preference and conditioned place aversion: effects of naloxone. *Psychopharmacology*. 1998; 139:62–70. [PubMed: 9768543]
- Cunningham C, Fidler T, Hill K. Animal models of alcohol's motivational effects. *Alcohol Res Health*. 2000; 24:85–92. [PubMed: 11199282]
- Cunningham CL, Ferree NK, Howard MA. Apparatus bias and place conditioning with ethanol in mice. *Psychopharmacology (Berl)*. 2003; 170:409–422. [PubMed: 12955296]
- Cunningham CL, Gremel CM, Groblewski PA. Drug-induced conditioned place preference and aversion in mice. *Nat Protoc*. 2006; 1:1662–1670. [PubMed: 17487149]
- Dayas CV, McGranahan TM, Martin-Fardon R, Weiss F. Stimuli linked to ethanol availability activate hypothalamic CART and orexin neurons in a reinstatement model of relapse. *Biol Psychiatry*. 2008; 63:152–157. [PubMed: 17570346]
- de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS II, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci USA*. 1998; 95:322–327. [PubMed: 9419374]
- Deng C, Li KY, Zhou C, Ye JH. Ethanol enhances glutamate transmission by retrograde dopamine signaling in a postsynaptic neuron/synaptic bouton preparation from the ventral tegmental area. *Neuropsychopharmacology*. 2009; 34:1233–1244. [PubMed: 18784647]
- Dole VP, Ho A, Gentry RT. Toward an analogue of alcoholism in mice: criteria for recognition of pharmacologically motivated drinking. *Proc Natl Acad Sci USA*. 1985; 82:3469–3471. [PubMed: 3858833]
- Fadel J, Deutch AY. Anatomical substrates of orexin–dopamine interactions: lateral hypothalamic projections to the ventral tegmental area. *Neuroscience*. 2002; 111:379–387. [PubMed: 11983323]
- Goldstein, DB. *Pharmacology of alcohol*. Oxford University Press, Oxford University Press; 1983.
- Gremel CM, Cunningham CL. Role of test activity in ethanol-induced disruption of place preference expression in mice. *Psychopharmacology (Berl)*. 2007; 191:195–202. [PubMed: 17216157]
- Gremel CM, Cunningham CL. Roles of the nucleus accumbens and amygdala in the acquisition and expression of ethanol-conditioned behavior in mice. *J Neurosci*. 2008; 28:1076–1084. [PubMed: 18234886]
- Gremel CM, Cunningham CL. Involvement of amygdala dopamine and nucleus accumbens NMDA receptors in ethanol-seeking behavior in mice. *Neuropsychopharmacology*. 2009; 34:1443–1453. [PubMed: 18830237]
- Gremel CM, Cunningham CL. Effects of disconnection of amygdala dopamine and nucleus accumbens *N*-methyl-D-aspartate receptors on ethanol-seeking behavior in mice. *Eur J Neurosci*. 2010; 31:148–155. [PubMed: 20092560]
- Groblewski PA, Bax LS, Cunningham CL. Reference-dose place conditioning with ethanol in mice: empirical and theoretical analysis. *Psychopharmacology (Berl)*. 2008; 201:97–106. [PubMed: 18633601]
- Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M, Sakurai T. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron*. 2001; 30:345–354. [PubMed: 11394998]
- Harris GC, Aston-Jones G. Arousal and reward: a dichotomy in orexin function. *Trends Neurosci*. 2006; 29:571–577. [PubMed: 16904760]
- Harris GC, Wimmer M, Aston-Jones G. A role for lateral hypothalamic orexin neurons in reward seeking. *Nature*. 2005; 437:556–559. [PubMed: 16100511]
- Harris GC, Wimmer M, Randall-Thompson JF, Aston-Jones G. Lateral hypothalamic orexin neurons are critically involved in learning to associate an environment with morphine reward. *Behav Brain Res*. 2007; 183:43–51. [PubMed: 17599478]
- Hollander JA, Lu Q, Cameron MD, Kamenecka TM, Kenny PJ. Insular hypocretin transmission regulates nicotine reward. *Proc Natl Acad Sci USA*. 2008; 105:19480–19485. [PubMed: 19033203]

- Ishii Y, Blundell JE, Halford JC, Upton N, Porter R, Johns A, Jeffrey P, Summerfield S, Rodgers RJ. Anorexia and weight loss in male rats 24 h following single dose treatment with orexin-1 receptor antagonist SB-334867. *Behav Brain Res.* 2005; 157:331–341. [PubMed: 15639184]
- Lawrence A, Cowen M, Yang H, Chen F, Oldfield B. The orexin system regulates alcohol-seeking in rats. *Br J Pharmacol.* 2006; 148:752–759. [PubMed: 16751790]
- Leeman RF, Heilig M, Cunningham CL, Stephens DN, Duka T, O'Malley SS. Ethanol consumption: how should we measure it? Achieving consilience between human and animal phenotypes. *Addict Biol.* 2010; 15:109–124. [PubMed: 20148775]
- Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, Elmquist JK. Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol.* 2001; 435:6–25. [PubMed: 11370008]
- Moorman DE, Aston-Jones G. Orexin-1 receptor antagonism decreases ethanol consumption and preference selectively in high-ethanol—preferring Sprague–Dawley rats. *Alcohol.* 2009; 43:379–386. [PubMed: 19671464]
- Narita M, Nagumo Y, Hashimoto S, Narita M, Khotib J, Miyatake M, Sakurai T, Yanagisawa M, Nakamachi T, Shioda S, Suzuki T. Direct involvement of orexinergic systems in the activation of the mesolimbic dopamine pathway and related behaviors induced by morphine. *J Neurosci.* 2006; 26:398–405. [PubMed: 16407535]
- Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS. Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci.* 1998; 18:9996–10015. [PubMed: 9822755]
- Pickering C, Avenon L, Liljequist S, Lindblom J, Schiöth HB. The role of hypothalamic peptide gene expression in alcohol self-administration behavior. *Peptides.* 2007; 28:2361–2371. [PubMed: 17976860]
- Plaza-Zabala A, Martin-Garcia E, de Lecea L, Maldonado R, Berrendero F. Hypocretins regulate the anxiogenic-like effects of nicotine and induce reinstatement of nicotine-seeking behavior. *J Neurosci.* 2010; 30:2300–2310. [PubMed: 20147556]
- Porter RA, Chan WN, Coulton S, Johns A, Hadley MS, Widdowson K, Jerman JC, Brough SJ, Coldwell M, Smart D, Jewitt F, Jeffrey P, Austin N. 1, 3-Biaryleureas as selective non-peptide antagonists of the orexin-1 receptor. *Bioorg Med Chem Lett.* 2001; 11:1907–1910. [PubMed: 11459658]
- Richards JK, Simms J, Steensland P, Taha SA, Borgland SL, Bonci A, Bartlett S. Inhibition of orexin-1/hypocretin-1 receptors inhibits yohimbine-induced reinstatement of ethanol and sucrose seeking in Long–Evans rats. *Psychopharmacology (Berl).* 2008; 199:109–117. [PubMed: 18470506]
- Risinger FO, Dickinson SD, Cunningham CL. Haloperidol reduces ethanol-induced motor activity stimulation but not conditioned place preference. *Psychopharmacology (Berl).* 1992; 107:453–456. [PubMed: 1615143]
- Rodgers RJ, Halford JC, Nunes de Souza RL, Canto de Souza AL, Piper DC, Arch JR, Upton N, Porter RA, Johns A, Blundell JE. SB-334867, a selective orexin-1 receptor antagonist, enhances behavioural satiety and blocks the hyperphagic effect of orexin-A in rats. *Eur J Neurosci.* 2001; 13:1444–1452. [PubMed: 11298806]
- Rustay NR, Crabbe JC. Genetic analysis of rapid tolerance to ethanol's incoordinating effects in mice: inbred strains and artificial selection. *Behav Genet.* 2004; 34:441–451. [PubMed: 15082941]
- Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell.* 1998; 92:573–585. [PubMed: 9491897]
- Schneider ER, Rada P, Darby RD, Leibowitz SF, Hoebel BG. Orexinergic peptides and alcohol intake: differential effects of orexin, galanin, and ghrelin. *Alcohol Clin Exp Res.* 2007; 31:1858–1865. [PubMed: 17850217]

- Sharf R, Sarhan M, Dileone RJ. Orexin mediates the expression of precipitated morphine withdrawal and concurrent activation of the nucleus accumbens shell. *Biol Psychiatry*. 2008; 64:175–183. [PubMed: 18423425]
- Sharf R, Guarnieri DJ, Taylor JR, DiLeone RJ. Orexin mediates morphine place preference, but not morphine-induced hyperactivity or sensitization. *Brain Res*. 2010; 1317:24–32. [PubMed: 20034477]
- Smart D, Sabido-David C, Brough SJ, Jewitt F, Johns A, Porter RA, Jerman JC. SB-334867-A: the first selective orexin-1 receptor antagonist. *Br J Pharmacol*. 2001; 132:1179–1182. [PubMed: 11250867]
- Smith RJ, See RE, Aston-Jones G. Orexin/hypocretin signaling at the orexin 1 receptor regulates cue-elicited cocaine-seeking. *Eur J Neurosci*. 2009; 30:493–503. [PubMed: 19656173]
- Smith RJ, Tahsili-Fahadan P, Aston-Jones G. Orexin/hypocretin is necessary for context-driven cocaine-seeking. *Neuropharmacology*. 2010; 58:179–184. [PubMed: 19591850]
- Xiao C, Shao XM, Olive MF, Griffin WC 3rd, Li KY, Krnjevic K, Zhou C, Ye JH. Ethanol facilitates glutamatergic transmission to dopamine neurons in the ventral tegmental area. *Neuropsychopharmacology*. 2009; 34:307–318. [PubMed: 18596684]

Fig. 1.

Mean activity counts/min ($\pm$ SEM) in 5-min bins during the 60 min after injection of SB-334867 in Experiment 1 (0, 10, 20, or 30 mg/kg; $n=12$ /dose group). The decrease in activity over time was significant, but there was no effect of dose

Fig. 2.

Mean time on the grid floor (s/min $\pm$ SEM) after two trials (*left panel*) and four trials (*right panel*) for each dose group in Experiment 2. On conditioning trials, mice in the G+ conditioning subgroups received ethanol (2 g/kg) injections immediately before 5-min exposure to the grid floor on CS+ trials; saline was injected before exposure to the hole floor on CS- trials. The cue-drug contingencies were reversed for mice in the G- conditioning subgroups. Mice had previously been pretreated with SB-334867 (0, 15, or 30 mg/kg) 30 min before each ethanol (CS+) conditioning trial. All mice were injected with saline before each test session ($n=14-16$ /conditioning subgroup). All dose groups showed significant CPP on both tests and CPP was stronger during the second test. However, there was no effect of dose during either test

Fig. 3.

Mean activity counts/min ($\pm$ SEM) during consecutive minutes of the ethanol (CS+) and saline (CS-) conditioning trials, averaged over all four trials of each type in Experiment 2. Mice were pretreated with SB-334867 (0, 15, or 30 mg/kg) 30 min before each ethanol (CS+) conditioning trial ($n=29-32$ /dose group). Activity on CS+ trials was significantly greater than activity on CS- trials. There were no differences between dose groups on CS- trials, but SB-334867 produced a dose-dependent reduction in ethanol-stimulated activity on CS+ trials. CS+ activity in the 30 mg/kg group was significantly less than CS+ activity in the 0 mg/kg vehicle control group ($p<0.001$); the 15 mg/kg group did not differ from either of the other groups

Fig. 4.

Mean time on the grid floor (s/min $\pm$ SEM) during the first 15 min of the 30-min test in Experiment 4. On conditioning trials, mice in the G+ conditioning subgroups received ethanol (2 g/kg) injections immediately before 5-min exposure to the grid floor on CS+ trials; saline was injected before exposure to the hole floor on CS- trials. The cue-drug contingencies were reversed for mice in the G- conditioning subgroups. Mice were pretreated with SB-334867 (0, 15, or 30 mg/kg) 30 min before the test, but received no pretreatment before conditioning trials ($n=30-32$ /conditioning subgroup). Two-way ANOVA confirmed development of significant CPP, but no effect of antagonist dose or interaction. Separate post hoc tests at each dose indicated a significant CPP in the 0 and 15 mg/kg groups (Bonferroni-corrected p values <0.001), but not in the 30 mg/kg group

Fig. 5.

Mean time on the grid floor (s/min $\pm$ SEM) after two trials (left panel) and four trials (right panel) for each treatment group in Experiment 5. On conditioning trials, mice in the G+ conditioning subgroups received ethanol (2 g/kg) injections immediately before 5-min exposure to the grid floor on CS+ trials; saline was injected before exposure to the hole floor on CS- trials. The cue-drug contingencies were reversed for mice in the G- conditioning subgroups. Mice were pretreated with SB-334867 (0 or 30 mg/kg groups) 30 min before Test 1 or received no pretreatment (No-Pretreat group). Mice were pretreated with vehicle (0 mg/kg group) or 40 mg/kg (No-Pretreat and 30 mg/kg groups) 30 min before Test 2. All mice had previously been injected with vehicle 30 min before each conditioning trial ($n=15-16$ /conditioning subgroup). Two-way ANOVAs confirmed development of significant CPP on both tests, but no effects of antagonist dose or interaction. The increase in CPP on the second test fell just short of the criterion for significance ($0.05 < p < 0.07$ for the interaction with test)

Table 1Mean activity counts per minute ($\pm$ SEM) on preference tests

Experiment	Treatment group (mg/kg)	Test after two trials	Test after four trials
2	0	34.8 $\pm$ 1.3	30.4 $\pm$ 1.5
	15	35.9 $\pm$ 1.2	32.0 $\pm$ 1.4
	30	36.1 $\pm$ 1.1	30.3 $\pm$ 1.1
4 ^a	0	NA	39.2 $\pm$ 1.3
	15	NA	41.1 $\pm$ 1.6
	30	NA	38.9 $\pm$ 1.5
5 ^b	No pretreat (40)	34.3 $\pm$ 1.0	26.4 $\pm$ 1.5
	0	31.5 $\pm$ 1.0	25.8 $\pm$ 1.3
	30 (40)	28.3 $\pm$ 0.8*	24.5 $\pm$ 1.4

^aData only from first 15 min of test for Experiment 4 (see text)^b40 indicates groups that received 40 mg/kg on the test after four trials* $p < 0.001$ (vs. no pretreat), Bonferroni-adjusted

Table 2

Mean blood ethanol concentration (mg/ml)

Group	10 min	30 min
Saline	2.3±0.07 (8)	2.1±0.04 (9)
0 mg/kg	2.1±0.08 (9)	2.2±0.10 (8)
30 mg/kg	2.0±0.08 (8) *	2.2±0.06 (9)

* $p = 0.04$ (vs. saline), Bonferroni-adjusted; group size in parentheses