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Genetic Versus Pharmacological Assessment of the Role of Cannabinoid Type 2 Receptors in Alcohol Reward-Related Behaviors

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Abstract

Background—Emerging evidence suggests that the endocannabinoid system (ECS) is involved in modulating the rewarding effects of abused drugs. Recently, the cannabinoid receptor 2 (CB2R) was shown to be expressed in brain reward circuitry and is implicated in modulating the rewarding effects of alcohol.

Methods—CB2 ligands and CB2R knockout (KO) mice were used to assess CB2R involvement in alcohol reward-related behavior in 2 well-established behavioral models: limited-access 2-bottle choice drinking and conditioned place preference (CPP). For the pharmacological studies, mice received pre-treatments of either vehicle, the CB2R agonist JWH-133 (10 and 20 mg/kg) or the CB2R antagonist AM630 (10 and 20 mg/kg) 30 minutes before behavioral testing. For the genetic studies, CB2R KO mice were compared to wild-type (WT) littermate controls.

Results—CB2R KO mice displayed increased magnitude of alcohol-induced CPP compared to WT mice. Neither agonism nor antagonism of CB2R affected alcohol intake or the expression of CPP, and antagonism of CB2R during CPP acquisition trials also did not affect CPP.

Conclusions—The CB2R KO CPP data provide partial support for the hypothesis that CB2Rs are involved in the modulation of alcohol reward-related behaviors. However, pharmacological manipulation of CB2Rs did not alter alcohol's rewarding effects in the alcohol-seeking models used here. These results highlight the importance of pharmacological validation of effects seen with lifetime KO models. Given the ongoing efforts toward medications development, future

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Conditioning trial activity for HAP2 mice in the experiments that tested effects of Rimonabant (A; $n = 79$), JWH-133 (B; $n = 95$), or AM630 (C; $n = 94$) on the expression of alcohol-induced CPP.

Fig. S2. Conditioning trial activity for CB2R KO ($n = 27$) and WT ($n = 24$) mice.

Fig. S3. Conditioning trial activity for C57BL/6 mice receiving Vehicle ($n = 12$), 10 mg/kg ($n = 11$) or 20 mg/kg ($n = 12$) AM630 prior to the alcohol conditioning trials.

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studies should continue to explore the role of the CB2R as a potential neurobiological target for the treatment of alcohol use disorders.

Keywords

Cannabinoid; CB2 Receptor; Alcohol; High-Alcohol-Preferring Mice; Reward

AN ESTIMATED 8.5 % of Americans suffer from alcohol use disorders (AUDs) in the United States. Estimated costs associated with AUDs exceed $223 billion and approximately 79,000 lives, with approximately 2.3 million years of potential life lost annually (Harwood, 2013). Because the burden of AUDs on society is so great, elucidating the mechanisms involved in the disease progression is critical. More specifically, identifying neurochemical systems involved in alcohol-related behaviors could pave the way for therapeutic targets that will ultimately reduce the burden of AUDs on society. One such system, the endocannabinoid system (ECS), has gained attention in recent years because it appears to play an important role in a variety of neuropsy-chiatric conditions (Abood and Martin, 1992; Hirst et al., 1998; Navarro and Rodriguez de Fonseca, 1998), including AUDs (see reviews by Justinova et al., 2009; Maldonado et al., 2006).

The ECS consists of 2 known receptor subtypes (cannabinoid receptor 1 [CB1R] and CB2R), 2 main endogenous ligands that act on these receptors (anandamide [AEA] and 2-arachidonoylglycerol [2-AG]), and the enzymes responsible for endocannabinoid degradation (monoacylglycerol lipase and fatty acid amide hydrolase). CB1R is highly expressed in brain reward circuitry and can regulate the excitatory and inhibitory inputs to the dopaminergic neurons of the mesocorticolimbic reward pathway (see review by Gardner, 2005). CB2Rs are found in a number of tissue and cell types in the periphery, including spleen, tonsils, and immune cells such as microphages and T cells. In the central nervous system, CB2Rs are expressed on neurons and activated microglia (Cabral et al., 2008; Garcia-Gutierrez et al., 2010; Onaivi, 2006). While there is still ongoing debate as to the localization of CB2R in the brain, there is evidence for expression in the caudate putamen, cingulate cortex, amygdala, hippocampus, ventromedial hypothalamic nucleus, arcuate nucleus, thalamus, substantia nigra, dorsal raphe nucleus, medial raphe nucleus, ventral tegmental area (VTA), and the nucleus accumbens (NAc) (Garcia-Gutierrez et al., 2010; Onaivi, 2006). The presence of CB2R in reward-related brain areas (i.e., NAc and VTA) suggests the possible involvement of this receptor in drug reward-related behaviors.

Both alcohol and activation of CB1R increase dopamine (DA) levels in the NAc, and cannabinoids (both endogenous and exogenous) can enhance the firing of dopaminergic neurons (Diana et al., 1998; French, 1997; French et al., 1997; Gessa et al., 1998; Hungund et al., 2003). Activation of CB2R has been shown to reduce cocaine-induced extracellular DA release and stimulated locomotor behavior (Xi et al., 2011), as well as reduce VTA DA neuronal firing (Zhang et al., 2014). These data, together with the observed localization of CB2Rs in the VTA, suggest that CB2Rs modulate drug reward-related behaviors.

Behavioral evidence further supports a role for CB2R in drug reward-related behaviors. Pharmacological activation of CB2Rs with JWH-133 dose-dependently reduced cocaine self-administration in wild-type (WT) and CB1R knockout (KO) mice, but not in CB2R KO

mice (Xi et al., 2011). Similarly, transgenic mice that overexpress CB2Rs self-administered less cocaine than WT controls (Aracil-Fernandez et al., 2012). CB2R KO mice showed reduced nicotine reward-related behaviors (Ignatowska-Jankowska et al., 2013; Navarrete et al., 2013) and pharmacological blockade of CB2Rs with SR144528 reduced nicotine-induced conditioned place preference (CPP) in C57BL/6 mice (Ignatowska-Jankowska et al., 2013).

Relevant to the current study with alcohol, Onaivi and colleagues (2008) showed reduced CB2R gene expression in the ventral midbrain region of inbred mice that developed a preference for alcohol compared to inbred mice that did not. In addition, chronic treatment with a CB2R agonist and a CB2R antagonist enhanced and reduced alcohol consumption, respectively, in stressed but not control inbred mice (Ishiguro et al., 2007; Onaivi et al., 2008). CB2R KO mice consumed more alcohol in an unlimited-access 2-bottle choice study and developed more robust alcohol-induced CPP compared to WT littermate controls (Ortega-Álvaro et al., 2015). Also, CB2R activation, via the agonist βcaryophyllene, reduced sensitivity to alcohol-induced sedation, alcohol intake, and alcohol-induced CPP in C57BL/6 mice (Al Mansouri et al., 2014). These findings are compelling and suggest that modulation of CB2Rs affects alcohol reward-related behaviors.

The purpose of this study was to assess the role of CB2R in modulating alcohol reward-related behaviors in 2 genetic mouse models and using 2 well-established models of alcohol reward: home cage limited-access 2-bottle choice alcohol drinking and alcohol-induced CPP. First, we pharmacologically assessed CB2R involvement in alcohol reward-related behaviors in selectively bred high-alcohol-preferring (HAP2) mice. Evidence suggests that genetic alterations in the ECS, including CB1R, may influence alcohol-related behaviors in rodents (e.g., Cippitelli et al., 2005; Hansson et al., 2007; Hungund and Basavarajappa, 2000). The HAP2 mouse line is a relevant model when studying genetically influenced mechanisms of AUDs in humans. We previously showed that HAP2 mice were more sensitive to pharmacological ECS manipulation compared to their low-alcohol-preferring counterparts (Powers et al., 2010), suggesting that genetic propensity toward high alcohol preference is associated with changes in ECS function. Second, we used mice with genetic deletion of CB2R (CB2R KO) to replicate the findings of Ortega-Álvaro and colleagues (2015) and to compare pharmacological versus genetic approaches in the study of the role of CB2R in alcohol reward-related behaviors. We hypothesized that pharmacological blockade and genetic deletion of CB2Rs would increase alcohol drinking and increase the expression of alcohol-induced CPP based on findings of Ortega-Álvaro and colleagues (2015), who showed that CB2R KO mice developed an alcohol-induced CPP, while WT controls did not.

MATERIALS AND METHODS

Subjects

For the pharmacological studies, male and female HAP mice from replicate line 2 (HAP2) were used. HAPs were produced by mass selection from outbred HS/Ibg mice (Institute for Behavioral Genetics, University of Colorado, Boulder, CO) at the Indiana Alcohol Research Center (IARC) in Indianapolis, IN (Grahame et al., 1999). Subjects in this study were alcohol-naïve and were generated at Purdue University from HAP2 (42nd generation)

breeders obtained from the IARC. Mice were between 80 and 160 days of age at the start of experimental procedures.

For the CB2R KO studies, male and female CB2R KO mice backcrossed to a C57BL/6J genetic background and their WT littermate controls (generously donated by Dr. Nancy Buckley, Cal State Polytechnic University, Pomona, CA) were used. Mice were between 82 and 152 days old when experiments started, and were allowed 12 days to acclimate to their new environment prior to the start of experimental procedures. All mice were alcohol-naïve for the CPP studies. Following CPP procedures, mice were given at least a 3-week washout period before the start of the alcohol drinking studies.

For the CPP acquisition study with AM630, male C57BL/6 mice were obtained from the Transgenic Mouse Core Facility at Purdue University, West Lafayette, IN. Mice were between 82 and 102 days old when the experiment began.

Drugs

For the CPP studies, alcohol was diluted from a 95% (v/v) solution to a concentration of 20% (v/v) with physiological saline (0.9%) and was administered intraperitoneally (IP) in a dose of 2.0 g per kilogram of body weight (g/kg; 0.06 g per 30 g body weight) and in an injection volume of 12.6 ml/kg. For the drinking study, alcohol was diluted from a 95% (v/v) solution to a concentration of 10% with tap water. Saccharin was added to both the alcohol and tap water drinking solutions at a concentration of 0.01% w/ v (Oberlin et al., 2010).

Rimonabant (Cayman Chemical, Ann Arbor, MI) was dissolved in 2 drops of Tween 80 and diluted with saline to the correct concentration to administer doses of 1 and 3 mg/kg, based on evidence that these doses effectively reduce alcohol intake in mice (Vinod et al., 2008). JWH-133 was received in Torcisolve (a water-soluble emulsion; Tocris Bioscience, Minneapolis, MN), diluted with saline, and administered in doses of 10 and 20 mg/kg, per Xi and colleagues (2011). AM630 (Cayman Chemical) was diluted in DMSO (10%) and saline (90%) and administered in doses of 10 and 20 mg/kg (10 ml/kg injection volume) to match the doses of JWH-133. All control (vehicle) solutions were made of the same ingredients as the drug solution, without the drug.

Place Conditioning Apparatus

The CPP apparatus consisted of 12 identical open-top plexiglass boxes enclosed in separate ventilated sound- and light-attenuating chambers, as previously described (Chester and Coon, 2010). The floor within each box is made up of 2 interchangeable halves with distinct floor textures (grid and/or hole). Side position within the box (left or right) and locomotor activity was monitored continuously during sessions for each mouse by the Hamilton-Kinder MotorMonitor program (Model HMM100; San Diego, CA).

Study Procedures

Limited-Access Drinking—The home cage limited-access drinking procedure is based on the work of Oberlin and colleagues (2010). Saccharin (0.01% w/v) was added to both the alcohol and water solutions to increase water consumption by HAP2 mice and thus reduce

alcohol preferences to around 60% in order to allow bidirectional detection of drug effects on alcohol intake.

Drinking procedures were conducted at the beginning of the dark phase of a reverse dark:light cycle (lights off at 09:45 hours). For 3 days before the start of drinking, mice were weighed and injected with saline (10 ml/kg volume) to habituate them to handling and injection procedures. On days 1 through 4 of alcohol availability, standard water bottles were replaced with serological pipettes fitted with stainless steel sipper tubes (measurement acuity 0.1 ml) containing either 10% (v/v) alcohol/saccharin or tap water/saccharin for 30 minutes. On day 4 (drug-testing day), CB2R drug pretreatments occurred 30 minutes before the presentation of alcohol.

For the studies in CB2R KO and WT mice, rimonabant (1 and 3 mg/kg) was given on the drug-testing day. This was carried out to pharmacologically probe CB1R in order to assess possible changes in CB1R sensitivity/function due to a lack of functional CB2Rs during development.

Conditioned Place Preference—The CPP studies involved a pretest, 4 conditioning trials with alcohol, and 1 posttest 24 hours after the fourth conditioning trial. Briefly, the pretest was used to assess animals' initial preference for the floor cues. Mice received an IP injection of saline (12.6 ml/kg) immediately before placement in the apparatus on a half grid/half hole floor for 60 minutes. Twenty-four hours later, mice received either alcohol 2 g/kg (IP) (CS+) or saline (CS−) on intervening days immediately before placement on a homogeneous floor type (grid or hole) for a 5-minute conditioning session. Floor type paired with alcohol or saline and CS+/CS− presentation order were completely counterbalanced. A 2-day break divided the first 2 and last 2 conditioning trials. Twenty-four hours following the final conditioning trial, mice were given an IP saline injection and tested for place preference during a 60-minute posttest, identical to the pretest.

Cannabinoid drugs were administered 30 minutes before, and a saline injection immediately before, the posttest (expression studies). The CPP acquisition study was identical to the expression studies except that C57BL/6 mice were given injections of 10 or 20 mg/kg AM630 or vehicle (10% DMSO in saline) 30 minutes before IP injection of alcohol (2 g/kg) on the CS+ days and vehicle 30 minutes before the saline injection on CS− days.

Statistical Analyses

For all studies, analysis of variance (ANOVA) in the Statistical Package for Social Sciences (SPSS; IBM Corp., Armonk, NY) was used. Between-subjects variables included sex (male and female), treatment (drug dose groups), genotype (CB2R KO and WT), and conditioning subgroup (grid+ and grid−). Within-subject factors were trials (1 to 4), conditioning session type (CS+ and CS−), average activity (s/min), and time (minutes) and day type (acquisition and drug-testing day). Significance was set at $p \leq 0.05$. Interactions and significant main effects were followed by lower-order ANOVAs and Dunnett's post hoc tests for treatment comparisons. Significant interactions containing the variables of interest (i.e., treatment and genotype) were explored with follow-up analyses.

Limited-Access Drinking—Alcohol (g/kg) and water intake (ml/ kg) were calculated and averaged for each mouse during acquisition (days 1 to 4) and compared to intake on the drug-testing day (day 5). This approach allowed for both within- and between-subjects analyses of alcohol intake.

Conditioned Place Preference—Time spent on the grid floor in each conditioning subgroup (grid+ and grid−) was the dependent variable used in the ANOVA for the analysis of CPP. Average activity (s/min) during conditioning and testing sessions was also assessed.

RESULTS

Pharmacological Studies

Limited-Access Drinking

Rimonabant (CB1R Antagonist)—Alcohol analyses (Fig. 1A): Repeated-measures ANOVA (Day Type × Sex × Treatment) on average g/kg alcohol intake (days 1 to 4) versus the test day (day 5) indicated Day Type × Treatment, $F(2, 43) = 3.4$, $p < 0.05$, and Day Type × Sex, $F(1, 43) = 15.0$, $p < 0.001$, interactions.

Follow-up analyses of day type within each treatment group yielded main effects ($Fs > 12.6$, $ps < 0.01$) due to reduced drinking in all treatment groups on the test day. Follow-up analyses of treatment group within each day type indicated no effects of treatment on acquisition days, but a main effect of treatment, $F(2, 48) = 5.8$, $p < 0.01$, on the test day. Dunnett's test showed that the 3 mg/kg dose of rimonabant significantly reduced alcohol intake compared to vehicle ($p < 0.001$) on the test day.

Water analyses—Three-way repeated-measures ANOVA on average water intake (ml/kg; days 1 to 4) versus the test day showed an interaction of Day Type × Treatment, $F(2, 43) = 3.6$, $p < 0.05$. Follow-up analyses of day type within each treatment group indicated a significant reduction in water intake in the 3 mg/kg group only, $F(1, 14) = 11.9$, $p < 0.01$. However, analyses of treatment within each day type showed no significant effects.

***JWH-133 (CB2R Agonist)—Alcohol analyses* (Fig. 1B):** Repeated-measures ANOVA (Day Type × Sex × Treatment) on g/kg alcohol intake (days 1 to 4) versus the test day indicated Day Type × Treatment, $F(2, 43) = 6.2$, $p < 0.01$, and Day Type × Sex, $F(1, 43) = 8.6$, $p < 0.01$, interactions.

Follow-up analyses of day type within each treatment group yielded main effects of day type for the vehicle, $F(1, 14) = 24.1$, $p < 0.001$, and 10 mg/kg groups, $F(1, 17) = 26.5$, $p < 0.01$, only.

Follow-up analyses of treatment group within each day type indicated a main effect of treatment, $F(2, 48) = 4.2$, $p < 0.05$, on the test day. Dunnett's test showed that the 20 mg/kg group had higher alcohol intake than the vehicle group ($p < 0.05$).

Water analyses: Three-way repeated-measures ANOVA on average water intake (ml/kg; days 1 to 4) versus the test day showed no significant effects.

***AM630 (CB2R Antagonist)—Alcohol analyses* (Fig. 1*C*):** Repeated-measures ANOVA (Day Type × Sex × Treatment) on average g/kg alcohol intake (days 1 to 4) versus the test day (day 5) indicated a Day Type × Sex interaction only, $F(1, 70) = 10.7$, $p < 0.01$.

Water analyses: Three-way repeated-measures ANOVA on average water intake (ml/kg; days 1 to 4) versus the test day showed no significant effects.

Expression of Alcohol-Induced CPP: Pretest preference was analyzed for all place conditioning studies. In all experiments, mice spent more time on the grid floor (32.0 to 37.0 s/min) versus the hole floor (23.0 to 28.0 s/min) on average during the 60-minute pretest, $Fs > 5.2$, $ps < 0.05$. To confirm that this floor bias would not affect interpretation of the results, 1-way ANOVAs (conditioning subgroup) were run on time on grid floor (averaged across the 60-minute pretest), which yielded no main effects of conditioning subgroup, indicating no evidence of a place preference prior to conditioning (ps > 0.2; data not shown). All conditioning trial activity data and analyses can be found in the Supplemental material.

***Rimonabant (CB1R Antagonist)—Posttest preference* (Fig. 2*A*):** Four-way ANOVA (Time × Sex × Conditioning Subgroup × Treatment) of time on grid floor yielded a main effect of conditioning subgroup only, $F(1, 46) = 26.8$, $p < 0.01$, indicating alcohol-induced CPP but no effect of rimonabant on the expression of alcohol-induced CPP.

Posttest activity: Three-way ANOVA (Time × Sex × Treatment) showed a Time × Sex interaction, $F(59, 4{,}307) = 1.4$, $p < 0.05$, due to faster decline in activity across the test session in males compared to females. There were no main effects or interactions with treatment (data not shown).

***JWH-133 (CB2R Agonist)—Posttest preference* (Fig. 2*B*):** Four-way ANOVA (Time × Sex × Conditioning Sub-group × Treatment) of time on grid floor yielded a main effect of conditioning subgroup, $F(1, 83) = 41.2$, $p < 0.01$, indicating alcohol-induced CPP but no effect of JWH-133 on the expression of alcohol-induced CPP.

Posttest activity: Three-way ANOVA (Time × Sex × Treatment) showed a main effect of time only, $F(59, 5{,}251) = 24.0$, $p < 0.01$, (data not shown).

***AM630 (CB2R Antagonist)—Posttest preference* (Fig. 2*C*):** Four-way ANOVA (Time × Sex × Conditioning Subgroup × Treatment) of time on grid floor showed a main effect of conditioning subgroup, $F(1, 82) = 79.7$, $p < 0.01$, indicating alcohol-induced CPP but AM630 did not affect the expression of alcohol-induced CPP.

Posttest activity: Three-way ANOVA (Time × Sex × Treatment) indicated a main effect of time only, $F(59, 5{,}192) = 23.8$, $p < 0.01$, (data not shown).

CB2R KO and WT Mice. Limited-Access Drinking—Al-cohol analyses (Fig. 3): Repeated-measures ANOVA (Day Type × Genotype × Sex × Treatment) on average g/kg alcohol intake (days 1 to 4) versus the test day (day 5) indicated Day Type × Treatment, $F(2, 40) = 4.2$, $p < 0.05$, and Day Type × Genotype × Sex, $F(1, 40) = 5.5$, $p < 0.05$, interactions.

Follow-up analyses of day type within each treatment group showed a main effect of day type in the 1 mg/kg, $F(1, 17) = 16.7$, $p < 0.01$, and the 3 mg/kg group, $F(1, 16) = 51.2$, $p < 0.001$, due to a decrease in alcohol intake on the test day.

Follow-up analyses of treatment group within each day type showed no effects on acquisition days. On the test day, there was a main effect of treatment, $F(2, 51) = 6.6$, $p < 0.01$, and follow-up Dunnett's test showed that the 3 mg/kg dose of rimonabant significantly reduced alcohol intake compared to vehicle in both genotypes ($p < 0.01$).

Water analyses: Three-way repeated-measures ANOVA on average water intake (ml/kg; days 1 to 4) versus the test day showed no significant effects.

Expression of Alcohol-Induced CPP—Posttest preference (Fig. 4): Four-way ANOVA (Time × Sex × Conditioning Subgroup × Genotype) of time on grid floor yielded a main effect of conditioning subgroup, $F(1, 43) = 7.4$, $p < 0.01$, indicating alcohol-induced CPP. A trend toward a Geno-type × Conditioning Subgroup interaction, $F(1, 43) = 2.5$, $p = 0.1$, was found; follow-up analyses showed a significant main effect of conditioning subgroup in KO mice only, $F(1, 24) = 8.3$, $p < 0.01$, similar to Ortega-Álvaro and colleagues (2015).

Posttest activity: Three-way ANOVA (Time × Sex × Genotype) indicated a Time × Genotype interaction, $F(59, 2{,}773) = 1.5$, $p = 0.01$. To explore this interaction, the data were collapsed into two 30-minute bins (first and last 30 minutes of the 60-minute session) and subjected to 1-way ANOVAs. Analyses indicated a significant main effect of genotype during the last 30 minutes only, $F(1, 49) = 6.7$, $p = 0.01$; WT > KO, but there was a similar trend for the first 30 minutes ($p = 0.09$; data not shown).

Effect of CB2R Antagonist (AM630) on the Acquisition of Alcohol-Induced CPP—: A follow-up study was conducted to assess the effect of administering AM630 during the acquisition phase of alcohol-induced CPP in C57BL/6 mice (background strain of CB2R KO mice). This study allowed for the comparison between effects of acute pharmacological blockade of CB2Rs during the conditioning trials versus effects of genetic deletion of CB2Rs on the magnitude of alcohol-induced CPP. Thus, this study addressed the possibility that developmental compensatory changes in the CB2R KO model could be responsible for the CB2R genotype difference in alcohol-induced CPP. Also, this study allowed for an assessment of the role of CB2Rs in the unconditioned (primary) rewarding effects of alcohol versus the expression of conditioned rewarding effects of alcohol.

***Posttest preference* (Fig. 5):** Three-way ANOVA (Time × Conditioning Subgroup × Treatment) of time on grid floor indicated a trend toward a main effect of Conditioning Subgroup only, $F(1, 29) = 2.7$, $p = 0.1$, indicating that AM630 did not affect the expression of alcohol-induced CPP, despite a visual trend (Fig. 5).

Posttest activity: Two-way ANOVA (Time × Treatment) indicated no significant effects (data not shown).

DISCUSSION

The goal of the present study was to assess the involvement of CB2Rs in alcohol reward-related behaviors in mice, using both genetic and pharmacological techniques and 2 well-established models of alcohol's motivational effects: limited-access 2-bottle choice drinking and alcohol-induced CPP. We hypothesized that genetic deletion and pharmacological blockade of CB2Rs would increase alcohol intake, while CB2R activation would decrease alcohol drinking, and that we would see similar effects for the expression of alcohol-induced CPP. Our results showed that neither activation nor blockade of CB2R affected either alcohol intake or the expression of alcohol-induced CPP. CB2R KO but not WT mice displayed significant alcohol-induced CPP. Pharmacological blockade of CB2R with AM630 during alcohol conditioning trials did not mimic the KO effect in C57BL/6 mice. Overall, inconsistencies between genetic and pharmacological results suggest that developmental compensatory changes in CB2R KO mice likely contribute to any genotype difference in alcohol reward-related behaviors.

We did not see increased alcohol drinking in CB2R KO compared to WT genotypes, consistent with Pradier and colleagues (2015) who showed no effect of genotype on alcohol intake using 3 models of 24-hour chronic alcohol consumption. However, the lack of a genotype effect is not consistent with a report by Ortega-Álvaro and colleagues (2015), who showed increased 24-hour drinking in CB2R KO mice compared to WT controls. One reason for these inconsistent findings could be related to the fact that the genetic background strain of the KO model in the Ortega-Álvaro and colleagues (2015) study was CD1 which are known to have low alcohol intake (Short et al., 2006). Another possibility is that CB2R KO mice have altered alcohol metabolism, but a recent study by Pradier and colleagues (2015) shows no difference in alcohol metabolism between genotypes. It should be noted that the drinking studies occurred 3 weeks after the CPP study and we cannot rule out possible changes in CB2R expression due to repeated alcohol exposure, as Onaivi and colleagues (2008) showed a down-regulation of CB2R in mice that developed a preference for alcohol consumption compared to mice that did not.

Although alcohol drinking was lower in many groups on the drug-testing day compared to intake during acquisition (Figs 1 and 3), it should be noted that intake on the test day in these HAP2 mice was still within a range previously shown to produce measurable blood alcohol levels within a 30-minute period based on comparison to HAP1 data reported in a study by Grahame and Grose (2003). Even so, we do not know whether the alcohol drinking behavior seen in this study produced blood alcohol levels that had physiologically relevant effects on the central nervous system. However, the low levels of alcohol intake did not preclude our ability to detect a drug effect (due to a floor effect, for example) because we were able to show that rimonabant reduced alcohol intake on the drug-testing day.

Overall, the activation of CB2Rs (JWH-133) did not alter alcohol drinking in HAP2 mice. However, between-subjects analyses indicated a significant group difference due to less of a reduction in alcohol intake on the test day (an effect which could be due to handling/injection stress effects) in the 20 mg/kg dose group than the 10 mg/kg dose and vehicle groups. This effect might simply be due to sampling error, but it is also possible that 20

mg/kg JWH-133 somehow prevented this decrease in alcohol intake seen in the other groups. If so, this finding might suggest that CB2Rs influence acute stress-induced changes in alcohol drinking. In fact, Ishiguro and colleagues (2007) reported that subchronic treatment with JWH-133 increased alcohol consumption in BALB/c mice following chronic mild stress (but not in non-stressed animals).

Pharmacological activation and inhibition of CB2Rs prior to the CPP preference test did not affect alcohol-induced CPP in HAP2 mice. In addition, inhibition of CB2Rs during conditioning trials in C57BL/6 mice, the same background strain as the KO mice, did not alter the acquisition of alcohol-induced CPP. CB2R KO mice showed greater alcohol-induced CPP than WT mice, replicating prior work (Ortega-Álvaro et al., 2015). The weak (nonsignificant) alcohol-induced CPP in the WT mice is not surprising given that the background strain is C57BL/6 which does not typically show a strong alcohol-induced CPP (Cunningham et al., 1992). The C57BL/6 mice we used for testing the effects of AM630 on the acquisition of alcohol-induced CPP showed a similar magnitude of CPP.

Compensatory biological mechanisms resulting from the absence of CB2Rs during development may explain the greater alcohol-induced CPP in CB2R KO mice. Ortega-Álvaro and colleagues (2015) reported increased μ-opioid receptor expression in the NAc of alcohol-naïve CB2R KO mice compared to WT mice, and acute alcohol administration increased μ-opioid receptor expression in CB2R KO mice compared to WT controls. The μ-opioid receptor plays an important role in the rewarding effects of alcohol (e.g., Gianoulakis, 2009; Herz, 1997) and future studies that examine opioid and cannabinoid system interactions in modulating alcohol-seeking behavior are warranted.

CB2R activation has been shown to decrease extracellular DA in the NAc in mice (Xi et al., 2011), and Kenny (2011) hypothesized that CB2Rs are expressed either directly on VTA DA neurons or on local GABAergic, glutamatergic, and/or cholinergic modulatory interneurons, indirectly influencing DA levels by affecting inhibition and excitation of DA neurons. Theoretically, the activation of CB2Rs on GABAergic interneurons (that exert inhibitory control over VTA dopaminergic neurons) could inhibit GABAergic control of VTA dopaminergic neuron firing, leading to increased DA release when alcohol is present. However, physiological evidence indicated that CB2R activation within the VTA reduces VTA DA neuronal firing (Zhang et al., 2014). Thus, 1 possible mechanism for the greater alcohol-induced CPP in CB2R KO mice compared to WT is that CB2Rs normally act to inhibit VTA DA neuronal firing and this results in lower DA activity and associated rewarding effects in response to alcohol. Therefore, CB2R KO mice may show increased alcohol-induced CPP because they lack the inhibitory control of CB2Rs.

Another possibility is that nonneuronal CB2Rs in the brain are modulating alcohol reward-related behaviors. Along with the recent evidence that CB2Rs are expressed on neurons in the VTA, more evidence shows that CB2Rs are expressed in the peripheral nervous system, and by other immune cells related to inflammation in the brain, including macrophages and microglia (see Cabral et al., 2008). More-over, the expression of CB2Rs is higher when microglia are in an activated state, suggesting the existence of a functional window where CB2Rs can modulate microglial activity (Cabral et al., 2008). Alcohol has been shown to

result in neuroinflamation and various immune responses in the brain (e.g., Pascual et al., 2007), and thus, CB2Rs expressed on neurons and/or immune cells in the brain could be important for modulating alcohol reward-related behaviors. Much more work is needed to elucidate the neurobiology underlying the behavioral effects of CB2R manipulation.

Onaivi and colleagues (2008) showed mice that developed a preference for alcohol had reduced CB2R expression levels in the ventral midbrain region compared to mice that did not develop a preference. Chronic treatment with a CB2R agonist and a CB2R antagonist enhanced and reduced alcohol consumption, respectively, in stressed but not control mice (Ishiguro et al., 2007; Onaivi et al., 2008). More recently, Xi and colleagues (2011) showed that CB2R activation decreased cocaine self-administration and Aracil-Fernandez and colleagues (2012) showed that genetic overexpression of CB2R decreased cocaine reward-related behaviors. In contrast, the rewarding effects of nicotine were reduced in mice with CB2R genetic deletion and CB2R antagonism (Ignatowska-Jankowska et al., 2013; Navarrete et al., 2013). Overall, these results suggest that the role of CB2Rs in drug reward-related behaviors is complex and depends on the drug being studied and whether genetic or pharmacological techniques are used. The current results highlight the importance of conflrming whether pharmacological manipulations mimic genetic KO models and whether similar effects are seen across mouse models with different genetic backgrounds.

In this study, we also assessed the role of CB1Rs in alcohol reward-related behaviors. The CB1R antagonist, rimonabant, was administered to HAP2 and KO/WT mice, and drinking was assessed to confirm that mice would show the expected reduction in alcohol intake. Rimonabant dose-dependently reduced alcohol intake in HAP2, CB2R KO, and WT mice, which is consistent with previous work in rats and inbred mice (e.g., Cippitelli et al., 2005; Colombo et al., 2007; Hansson et al., 2007; Hungund et al., 2003). Rimonabant reduced alcohol intake to the same degree in both CB2R KO and WT mice, suggesting that there are likely no developmental compensatory changes in CB1Rs in the CB2R KO model.

Further, we tested the effects of rimonabant on the expression of alcohol-induced CPP and found no effect. These data are consistent with those of Pina and Cunningham (2014) who showed no effect of the novel CB1R antagonist, PF 514273, on the expression (or acquisition) of alcohol-induced CPP in DBA/2J mice. A noted limitation of their study was that PF 514273 was a novel compound and dosage and schedule of administration were based on only 1 published in vivo study. Our results and those of Pina and Cunningham (2014) suggest that CB1Rs do not influence alcohol's primary or conditioned rewarding effects in the place conditioning procedure. The discrepancy between rimonabant's effects in oral self-administration versus place conditioning procedures could be due to the well-known effects of rimonabant in suppressing general consummatory behavior (e.g., Freedland et al., 2001) and appetite (Fong and Heymsfield, 2009). More work will be needed to understand the role of the CB1Rs (and their interaction with CB2Rs) in modulating the motivational effects of alcohol.

In summary, CB2R KO mice showed greater alcohol-induced CPP compared to their WT counterparts, similar to Ortega-Álvaro and colleagues (2015). However, we were not able to mimic this effect through pharmacological blockade of CB2Rs during the acquisition of CPP

in C57BL/6 mice, the same background strain of the KO model. Pharmacological activation/inhibition of CB2Rs did not alter alcohol drinking or the expression of alcohol-induced CPP. Overall, these findings provide partial support for the hypothesis that CB2Rs are involved in the modulation of alcohol reward-related behaviors, but suggest that targeting CB2Rs, at least with the current pharmacological tools available, does not alter alcohol's rewarding effects in the alcohol-seeking models used here. Our results highlight the importance of pharmacological validation of effects seen with lifetime KO models. Given the ongoing efforts toward medications development, future studies should continue to explore the role of the CB2 receptor as a potential neurobiological target for the treatment of alcohol use disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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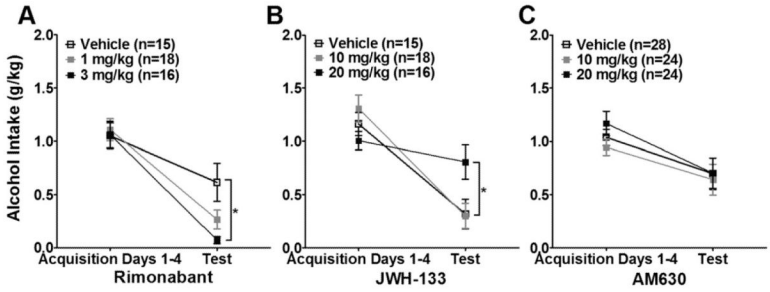

Fig. 1.

(A) Mean (±SEM) change in alcohol intake (g/kg) between acquisition days 1 to 4 and the test day in HAP2 mice (n = 15 to 18) pretreated with vehicle, 1 mg/kg rimonabant, or 3 mg/kg rimonabant. *p < 0.001, vehicle > 3 mg/kg rimonabant. (**B**) Mean (±SEM) change in alcohol intake (g/kg) between acquisition days 1 to 4 and the test day in HAP2 mice (n = 15 to 18) pretreated with vehicle, 10 mg/kg JWH-133, or 20 mg/kg JWH-133. *p < 0.05, 20 mg/kg JWH-133 > vehicle. (**C**) Mean (±SEM) change in alcohol intake (g/kg) between acquisition days 1 to 4 and the test day in HAP2 mice (n = 24 to 28) pretreated with vehicle, 10 mg/kg AM630, or 20 mg/kg AM630.

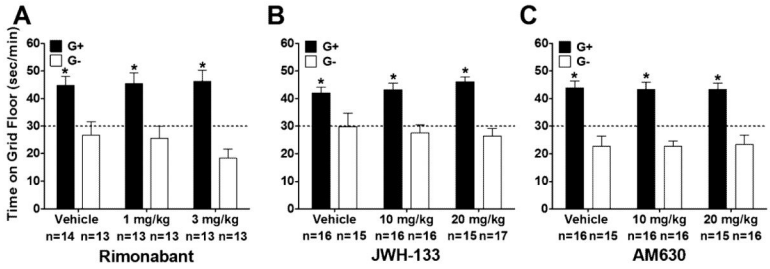

Fig. 2.

(A) Mean (±SEM) raw time (s/min) spent on the grid floor in G+ and G− conditioning subgroups (n = 13 to 14 per subgroup) during the posttest for HAP2 mice pretreated with vehicle, 1 mg/kg rimonabant, or 3 mg/kg rimonabant. *$p < 0.01$, main effect of G+ > G−. (**B**) Mean (±SEM) raw time (s/ min) spent on the grid floor in G+ and G− conditioning subgroups (n = 15 to 17 per subgroup) during the posttest for HAP2 mice pretreated with vehicle, 10 mg/kg JWH-133, or 20 mg/kg JWH-133. *$p < 0.01$, main effect of G+ > G−. (*C*) Mean (±SEM) raw time (s/min) spent on the grid floor in G+ and G− conditioning subgroups (n = 15 to 16 per subgroup) during the posttest for HAP2 mice pretreated with vehicle, 10 mg/kg AM630, or 20 mg/kg AM630. *$p < 0.01$, main effect of G+ > G−.

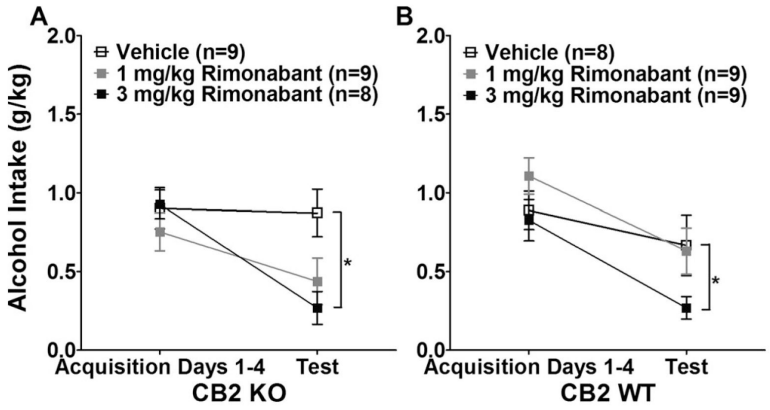

Fig. 3.

(A) Mean (±SEM) change in alcohol intake (g/kg) between acquisition days 1 to 4 and the test day in cannabinoid receptor 2 (CB2R) knockout (KO) mice (n = 8 to 9) pretreated with vehicle, 1 mg/kg rimonabant or 3 mg/kg rimonabant. (**B**) Mean (±SEM) change in alcohol intake (g/kg) between acquisition days 1 to 4 and the test day in cannabinoid receptor 2 (CB2R) wild-type (WT) mice (n = 8 to 9) pretreated with vehicle, 1 mg/kg rimonabant, or 3 mg/kg rimonabant. *$p < 0.05$, main effect of vehicle > 3 mg/kg rimonabant across genotypes.

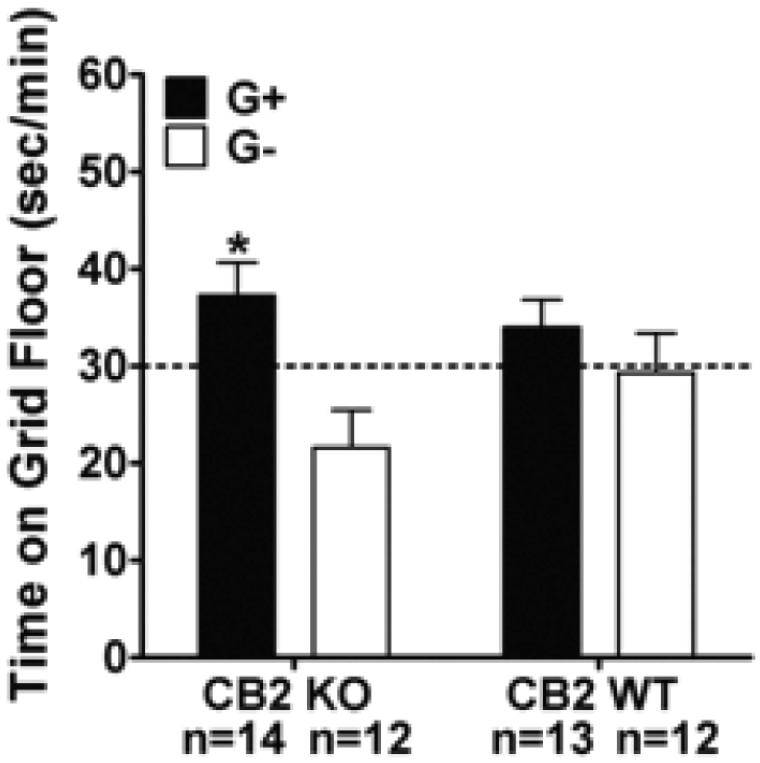

Fig. 4.

Mean (±SEM) raw time (s/min) spent on the grid floor in G+ and G− conditioning subgroups (n = 12 to 14 per subgroup) during the posttest for cannabinoid receptor 2 (CB2R) knockout (KO) and wild-type (WT) mice. *$p < 0.05$, G+ > G−.

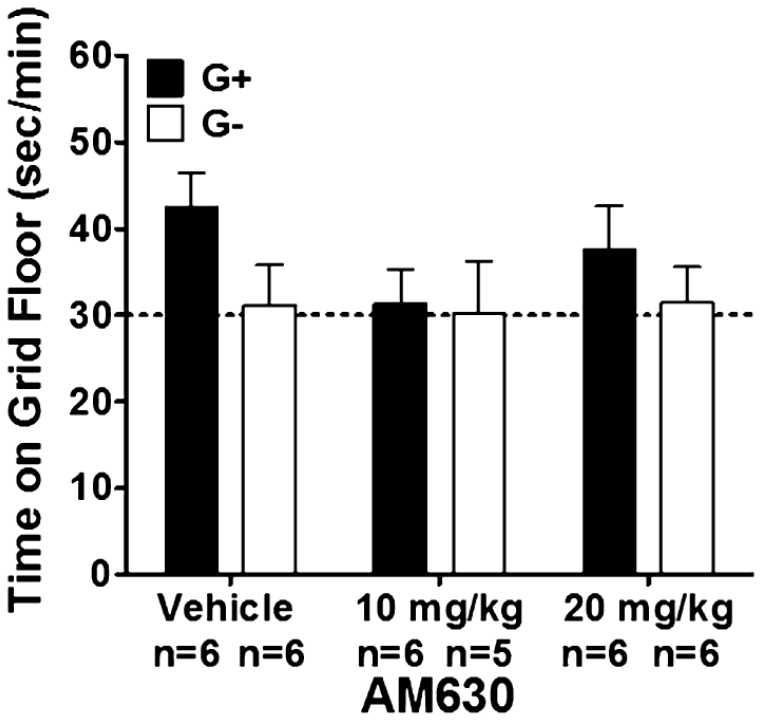

Fig. 5.

Mean (±SEM) raw time (s/min) spent on the grid floor in G+ and G− conditioning subgroups (n = 5 to 6 per subgroup) during the posttest for HAP2 mice pretreated with vehicle, 10 mg/kg AM630, or 20 mg/kg AM630 before CS+ conditioning trials.