Pharmacology, Biochemistry and Behavior 124 (2014) 260–268

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

The cannabinoid receptor 2 agonist, β-caryophyllene, reduced voluntary alcohol intake and attenuated ethanol-induced place preference and sensitivity in mice

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ARTICLE INFO

Article history:
Received 18 May 2014
Received in revised form 15 June 2014
Accepted 27 June 2014
Available online 3 July 2014

Keywords:
AM630
β-Caryophyllene
CB_2 receptors
Conditioned place preference
LORR
Two-bottle choice

ABSTRACT

Several recent studies have suggested that brain CB_2 cannabinoid receptors play a major role in alcohol reward. In fact, the implication of cannabinoid neurotransmission in the reinforcing effects of ethanol (EtOH) is becoming increasingly evident. The CB_2 receptor agonist, β-caryophyllene (BCP) was used to investigate the role of the CB_2 receptors in mediating alcohol intake and ethanol-induced conditioned place preference (EtOH-CPP) and sensitivity in mice. The effect of BCP on alcohol intake was evaluated using the standard two-bottle choice drinking method. The mice were presented with increasing EtOH concentrations and its consumption was measured daily. Consumption of saccharin and quinine solutions was measured following the EtOH preference tests. Finally, the effect of BCP on alcohol reward and sensitivity was tested using an unbiased EtOH-CPP and loss of righting-reflex (LORR) procedures, respectively. BCP dose-dependently decreased alcohol consumption and preference. Additionally, BCP-injected mice did not show any difference from vehicle mice in total fluid intake in a 24-hour paradigm nor in their intake of graded concentrations of saccharin or quinine, suggesting that the CB_2 receptor activation did not alter taste function. More importantly, BCP inhibited EtOH-CPP acquisition and exacerbated LORR duration. Interestingly, these effects were abrogated when mice were pre-injected with a selective CB_2 receptor antagonist, AM630. Overall, the CB_2 receptor system appears to be involved in alcohol dependence and sensitivity and may represent a potential pharmacological target for the treatment of alcoholism.

1. Introduction

Alcohol dependence is a serious medical, social and economic problem in terms of morbidity, mortality and disability worldwide. According to the World Health Organization (WHO), alcohol dependence is responsible for 4% of global disease which is slightly lower than that caused by smoking (4.1%) and hypertension (4.4%) (WHO, 2004). In recent years, accumulating evidence suggests an interaction between alcohol dependence and the endocannabinoid system (ECS) wherein the endogenous bioactive lipid-derived endocannabinoid ligands act through the cannabinoid receptors CB_1 and CB_2, which couple to the $G\alpha_{i/o}$ class of G-proteins and have presynaptic or postsynaptic distribution in the brain (Breivogel and Childers, 1998). They also serve as retrograde transmitters in central synapses. The ECS has been shown to play an important role in drug abuse and dependence (Maldonado et al., 2006) including the reinforcing effects of alcohol (Erdozain and Callado, 2011), opioids (Manzanedo et al., 2004), nicotine (Viveros et al., 2007) and cocaine (Tanda, 2007). Since the discovery of ECS, accumulating evidence has demonstrated that alcohol interacts with ECS and the cannabinoid receptors CB_1 and CB_2 play an important and pervasive role in the etiology of alcohol dependence suggesting that these receptors could be a potential therapeutic target (Basavarajappa, 2007; Onaivi, 2009). The pharmacological blockade or genetic ablation of CB_1 receptors shows decreased operant self-administration of EtOH and voluntary consumption of EtOH in rodents (Onaivi, 2009; Onaivi et al., 2008a; Vinod et al., 2008). Numerous preclinical studies have demonstrated that activation of CB_1 receptor facilitates EtOH consumption (Klugmann et al., 2011) while antagonism of CB_1 receptor reduces the motivational properties of EtOH with no effect

Abbreviations: BCP, β-caryophyllene; CPP, conditioned-place preference; EtOH, ethanol; BEC, blood ethanol concentration; ECS, endocannabinoid system; LORR, loss of righting reflex.

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http://dx.doi.org/10.1016/j.pbb.2014.06.025
0091-3057/

on EtOH metabolism (Basavarajappa, 2007). Even though CB_1 antagonism appeared to be favorable for the treatment of alcohol dependence, reports of the adverse psychiatric effects with CB_1 antagonists in clinical trials have directed the development of other alternatives in ECS, especially CB_2 receptors identified in the brain particularly in the cortex, hippocampus and substantia nigra (Brusco et al., 2008; Onaivi et al., 2006) and also involved in the anxiogenic, pneumonic and motor processes (Atwood and Mackie, 2010).

In addition, alcohol consumption is known to alter CB_2 receptor gene expression in the brain (Onaivi et al., 2008b). Therefore, given the functional relevance of the CB_2 receptor, its pharmacological targeting may provide a novel therapeutic avenue for the treatment of alcohol dependence. Activation of the CB_2 receptors appears to be an interesting pharmacological strategy for circumventing the unwanted psychotropic effects of CB_1 receptor inhibition (Maccioni et al., 2010). In a recent study, the selective activation of CB_2 receptors was shown to reduce the reinforcing effects of cocaine (Xi et al., 2011). Currently, most drug development in the CB_2 field of research has focused on the development of CB_2 receptor agonists of synthetic and natural origin. Considering the need for novel compounds for their therapeutic potential in alcohol dependence, there is a renewed interest in the bioactive agents of natural origin.

Recently, β-caryophyllene (BCP), a naturally-derived sesquiterpene possessing full agonist activity on CB_2 receptors (Ki values of 155 nmol/l for human CB_2 receptors with no significant affinity for binding to CB_1 receptors) has garnered attention for its therapeutic potential (Gertsch et al., 2008, 2010). The recent approval of BCP by the FDA as a food additive and in cosmetics, as well as its widespread availability and apparent non-toxicity (generally regarded as safe), makes it an excellent candidate for a novel therapeutic approach in alcohol dependence (Gertsch et al., 2008). This is especially true because the majority of the therapeutic activities and underlying pharmacological mechanisms of BCP (Bento et al., 2011; Horvath et al., 2012) have been shown to be attributed to its CB_2 receptor selective-agonist activity.

Therefore, in the present study, the aim was to investigate the effects of BCP on alcohol consumption and evaluate its preference in mouse models of alcohol addiction. Specifically, we examined alcohol appetitive behaviors in a two-bottle choice paradigm using an unlimited EtOH access paradigm in vehicle- and BCP-treated C57BL/6 adult male mice. We then examined several factors involved in mediating alcohol appetitive behaviors including sweet/bitter taste sensitivity, EtOH-elicited CPP and EtOH sensitivity using LORR. Finally, to elucidate the CB_2 receptor-mediated mechanism and assess the contribution of this receptor in the anti-addictive action of BCP, we examined the effects of AM630, a pharmacological antagonist of CB_2 receptor that was given prior to BCP treatment.

2. Materials and methods

2.1. Animals

All experiments were performed on adult male C57BL/6 mice which were obtained from the local breeding facility of the College of Medicine & Health Sciences (CMHS), UAE University. The animals were maintained in a temperature-controlled (~22 °C) colony room with a 12-h light–dark cycle (0600–1800 light on) and food was available *ad libitum* throughout the experiment. Standard rodents' chow diet was obtained from the National Feed and Flour Production and Marketing Company LLC (Abu Dhabi, UAE). The experiment began 10 days after animal procurement at which time the mice were housed singly in standard Plexiglas observation cages. All the experimental procedures were approved by the local Institutional Animal Care and Use Committee (Approval No. A25-13).

2.2. Drugs

Absolute EtOH was obtained from Panreac Quimica SAU (Barcelona, Spain) and diluted into 2.5–20% solutions (*v*/*v*) using ordinary tap water. For taste sensitivity, saccharin sodium salt dihydrate (0.04 and 0.08%; *w*/*v*) and quinine hemisulfate (30 and 60 μM; *w*/*v*) were purchased from Sigma-Aldrich (MO, USA) and dissolved in tap water. For EtOH-induced conditioned place preference (EtOH-CPP), alcohol was diluted in isotonic saline 0.9% NaCl (10%; *v*/*v*). The test drug, BCP (25, 50 and 100 mg/kg), was diluted in olive oil. However, the selective antagonist of CB_2 receptors, AM630 (3 mg/kg) obtained from Sigma-Aldrich was diluted in 2.5% DMSO. Both the drugs and solvents were obtained from Sigma-Aldrich and injected intraperitoneally (i.p.) with a volume of 10 ml/kg adjusted to body weight. For EtOH, saccharin and quinine intake, vehicle, BCP and AM630 the animals were injected daily 30 min before the lights were switched off.

2.3. Two-bottle choice: EtOH consumption and preference

A continuous, two-bottle choice paradigm was used to compare EtOH consumption and preference between vehicle- and BCP-injected mice as described previously (Bahi, 2012, 2013a). Table 1 shows the general design of the study. In brief, during the acclimatization period mice were presented with two 10 ml graduated pipettes fitted with stainless-steel drinking spouts containing only water. At the onset of the test, one of the pipettes containing water was replaced with a 2.5% alcohol solution. Every 4 days the alcohol solution was replaced with a 5%, 10% and finally 20% alcohol solution. The position of the pipettes in each cage was alternated daily to control for side preference and daily fluid intake was recorded from both pipettes. To obtain an accurate measurement of EtOH consumption, the amount of daily alcohol consumption was determined (in g/kg of body weight) for each animal and averaged over the 4-day period for each EtOH concentration. EtOH preference was calculated by dividing the volume of EtOH solution consumed by the total volume of EtOH and water consumed [(EtOH intake / EtOH + water intake) × 100]. Total intakes from both bottles were calculated to obtain total fluid intake per kilogram of body weight for each subject. Two control pipettes (one with water and the other with the corresponding alcohol solution) were used to estimate evaporation and spillage and the consumption amounts obtained for experimental animals were adjusted accordingly. Mouse body weights were measured every 4th day.

2.4. Two-bottle choice: sweet/bitter tastant consumption and preference

To check for differences in taste sensitivity, consumption and preference of saccharin and quinine solutions were assessed following the

Table 1
Summary of the experimental procedure used in the two-bottle choice test.

Fluids	Days
Baseline water drinking[a]	7
2.5% ethanol *vs.* water[b]	4
5% ethanol *vs.* water[b]	4
10% ethanol *vs.* water[b]	4
20% ethanol *vs.* water[b]	4
Washout/water[a]	7
0.04% saccharin *vs.* water[b]	4
0.08% saccharin *vs.* water[b]	4
Washout/water[a]	7
30 μM quinine *vs.* water[b]	4
60 μM quinine *vs.* water[b]	4
Washout/water[a]	7
10% ethanol *vs.* water[b]	4

[a] Tap water was available in both drinking bottles.
[b] In all the two-bottle tests, the drinking bottles were rotated daily to prevent position preference.

EtOH intake test as described previously (Bahi, 2013b; Bahi and Dreyer, 2012a). Seven days after the final day of alcohol consumption, the mice were given water and a non-alcoholic solution containing either a sweet (saccharin: 0.04 and 0.08%) or a bitter (quinine: 30 and 60 μM) tastant as before (for 4 days with escalating concentrations as the low concentration was always presented first, followed by the higher concentration). The quantity of saccharin and quinine consumed (grams per kilogram of body weight per 24 h) was calculated for each mouse and these values were averaged for each concentration of tastant. As before, the mice were weighed every 4th day, the intake of each solution was determined daily and the pipette position was alternated daily to control for any potential side preference. There was a 1-week water-only period between testing the saccharin and quinine solutions.

In the 1st experiment, the mice were injected daily with vehicle (n = 13) or BCP (50 mg/kg, i.p.) (n = 14) using increasing concentrations of either alcohol [2.5, 5, 10 and 20% in tap water (*v*/*v*)] or tastants (saccharin 0.04 and 0.08% or quinine 30 or 60 μM). The concentrations were increased every 4th day and fluid intake was measured 24 h later as described above. To show that tolerance did not develop to BCP the same mice were injected daily with vehicle or BCP (50 mg/kg, i.p.) while given access to 10% ethanol solution for 4 days and ethanol intake and preference were measured as described above.

In the 2nd experiment, a group of naïve mice (n = 13) was injected with increasing doses of BCP (0, 25, 50 and 100 mg/kg, i.p.) to determine its effect on EtOH consumption and preference for a solution of 10% EtOH *versus* ordinary tap water. For this purpose, the naïve mice received daily BCP injections, 20 min before the light was switched off. BCP doses were increased every 4th day, while fluid levels in each pipette and quantities consumed were measured 24 h later as described above.

2.5. EtOH-induced, conditioned place preference

The effect of BCP on EtOH-CPP acquisition was performed as described previously (Bahi and Dreyer, 2012b; Bahi et al., 2013a). In brief, the two-chamber CPP apparatus contained two wooden-conditioning chambers (300 mm × 300 mm × 300 mm), which offered the mice different tactile and visual cues (wall color and floor). Both chambers could be accessed through a 10 cm guillotine doorway-like opening. The time spent in each chamber was measured.

2.5.1. Pre-conditioning

On day 0, a baseline pretest was performed to determine the preference of each mouse; all mice received saline injections (10 ml/kg, i.p.) and were placed between the two chambers and allowed to explore freely the apparatus for 15 min. During this session, mice exhibiting unconditioned preference (more than 70%) or aversion (less than 30%) for any compartment were discarded from the conditioning session. For each experimental group, the average time (in seconds) spent in the black chamber was as follows [saline conditioning: DMSO–Oil (433.50 ± 29.29), DMSO–BCP (431.38 ± 18.72), AM630–Oil (445.00 ± 22.75) and AM630–BCP (437.67 ± 17.49)] and [EtOH conditioning: DMSO–Oil (450.18 ± 19.99), DMSO–BCP (441.67 ± 15.36), AM630–Oil (438.14 ± 22.95) and AM630–BCP (461.07 ± 16.24)]. Therefore, the mice did not show an unconditioned preference for either of the chambers which supported the use of an unbiased method. Consequently, in one of the chambers black or white, randomly chosen, the mice received saline and in the other they were injected with EtOH.

2.5.2. Conditioning

During the conditioning days 1–5, mice were given twice-daily 30 min sessions in the apparatus. The EtOH CPP group was confined to one conditioning chamber after saline injection in the morning; after 6 h, EtOH CPP mice were given an injection of EtOH (1.5 g/kg, i.p. given from 10% EtOH solution) and placed in the other conditioning chamber. The saline CPP group received saline injections before being placed in either chamber. The pairings with EtOH happened in a balanced manner, *i.e.*, approximately half to the white and half to the black compartment. BCP (with or without AM630) was injected 15 min before EtOH conditioning. This pretreatment–treatment combination created four test groups: [saline conditioning: DMSO–Oil (n = 10), DMSO–BCP (n = 13), AM630–Oil (n = 12) and AM630–BCP (n = 12)] and [EtOH conditioning: DMSO–Oil (n = 11), DMSO–BCP (n = 15), AM630–Oil (n = 14) and AM630–BCP (n = 15)].

2.5.3. Post-conditioning

On day 6, expression phase tests were performed; each mouse was given a saline injection (10 ml/kg, i.p.) and placed in the center of the CPP apparatus with free access to both conditioning chambers for 15 min. The time spent by each mouse in the EtOH-paired side was monitored.

2.6. EtOH sensitivity/loss or righting reflex (LORR)

EtOH-naïve male mice were tested for their sensitivity to EtOH with a LORR test. Briefly, mice were injected with sedative doses of EtOH (3, 3.4 or 3.8 g/kg EtOH from 20% solution in isotonic saline). Ten minutes after the injection, all mice were put in a supine position in a plastic V-shaped trough and tested to ensure presence of the righting reflex. The time of EtOH-induced LORR (sleep time, time elapsed between the onset of sedation and righting of mice back on all four paws) was recorded. The recovery was defined as the time required for mice to right themselves three times in 30 s after being placed on their backs. To test the effect of CB_2 receptor activation on LORR, mice were given BCP (50 mg/kg, i.p.) alone or after AM630 (3 mg/kg). Fifteen minutes later, the mice were injected with EtOH and LORR latency was measured as described above. All of the experiments were performed at room temperature with the EtOH injection occurring between 11 am and 2 pm. This pretreatment–treatment combination created four test groups: DMSO–Oil (n = 7), DMSO–BCP (n = 7), AM630–Oil (n = 7) and AM630–BCP (n = 7).

2.7. Blood ethanol concentration (BEC)

Naïve, adult mice (n = 4 for each group tested in duplicate) were used to test the effect of vehicle or BCP (50 mg/kg) on blood alcohol levels when given an equal amount of EtOH (grams per kilogram of body weight) as described previously (Bahi, 2013b; Bahi et al., 2013a, b). Accordingly, mice were treated with a 3 g/kg dose of EtOH (i.p.; 20% *v*/*v* in isotonic saline) and for the 1st group (n = 4), trunk blood was collected from each mouse 3 h after injection. For the 2nd group (n = 4), blood collection was performed 6 h after injection. The blood samples were centrifuged at 3000 rpm for 10 min and 5 μl of plasma from each sample was analyzed for BECs using an alcohol dehydrogenase assay kit (BioVision Research Products, CA, USA) following the manufacturer's protocol. The BEC was expressed in nmol/μl.

2.8. Statistical analysis

For statistical comparisons, the software package IBM SPSS Statistics 21 was used. Data were expressed as mean values ± SEM. The data representing the effects of BCP on EtOH and tastant consumption and preference were analyzed using a two-way repeated-measure analysis of variance (ANOVA) with drug as the between-subjects factor and concentration as the within-subject factor. The effect of BCP and AM630 on the EtOH-CPP was analyzed using two-way ANOVA with repeated measure with drug and conditioning (EtOH or saline) as the between-subjects factors and time (pre- and post-conditioning) as the within-subject factor. The data representing EtOH-induced LORR was analyzed using two-way ANOVA with the

different drugs and EtOH concentration as the between-subjects factors. Finally, the data representing effect of BCP on BEC were analyzed using a one-way ANOVA repeated measure with drug as the between-subjects factor and time as the within-subject factor. In case of a significant main effect, post hoc comparisons were performed with Bonferroni's test. The criterion for statistical significance was set at $P \leq 0.05$.

3. Results

3.1. BCP attenuated EtOH consumption and preference in mice

To determine the impact of BCP on voluntary EtOH intake and preference, we performed a continuous access two-bottle-choice drinking test. It should be emphasized that a single injection of BCP did not produce a significant effect on ethanol intake (data not shown). Therefore, we performed repeated daily injections of BCP and ethanol consumption and preference were measured. Fig. 1 shows the consumption and preference for EtOH of vehicle- and BCP (50 mg/kg)-injected mice with increasing concentrations of alcohol (2.5, 5, 10 and 20%; *v*/*v*). As depicted in Fig. 1A, a two-way ANOVA with drug (vehicle and BCP) and EtOH as the between-subjects factors showed that, compared to vehicle-, BCP-treated mice displayed decreased EtOH consumption as revealed by a main effect of drug ($F_{(1,25)} = 26.589$, $P \leq 0.001$). In addition, statistical analysis revealed a main effect of EtOH concentration ($F_{(3,75)} = 120.176$; $P \leq 0.001$). More importantly, the interaction between drug treatment and EtOH concentration was statistically significant ($F_{(3,75)} = 7.165$; $P \leq 0.001$). As expected, the activation of CB_2 receptors by BCP also reduced EtOH preference. In fact and as depicted in Fig. 1B, there was a main effect of drug treatment ($F_{(1,25)} = 14.001$; $P = 0.001$) and EtOH concentration ($F_{(3,75)} = 25.302$; $P \leq 0.001$) on alcohol preference. Importantly, the drug × EtOH concentration interaction was also significant ($F_{(3,75)} = 8.485$; $P \leq 0.001$). Finally, relative total fluid consumption was not affected following BCP injection. In fact, there was no main effect of drug ($F_{(1,25)} = 1.545$; $P = 0.225$) nor EtOH concentration ($F_{(3,75)} = 1.232$; $P = 0.304$) on total fluid intake. Furthermore, the interaction between drug and EtOH concentration was not significant ($F_{(3,75)} = 0.583$; $P = 0.628$) (Fig. 1C). These findings suggest that the decreased EtOH consumption and preference following BCP injection were not caused by an overall decrease in total amount of fluid consumed.

3.2. Vehicle- and BCP-injected mice do not show differences in tastant intake and preference

To determine whether differences in alcohol consumption and preference might reflect changes in taste neophobia caused by BCP injection, drinking studies with saccharin and quinine were performed. Fig. 2A shows consumption (grams per kilogram) in vehicle- and BCP-injected mice. Two-way ANOVA with repeated measures identified a significant effect of saccharin concentration ($F_{(1,25)} = 312.717$, $P \leq 0.001$), but no effect of drug ($F_{(1,25)} = 0.109$, $P = 0.744$) or significant interaction of drug and saccharin concentration ($F_{(1,50)} = 0.005$, $P = 0.943$). Similarly, there were no differences between groups in preference for saccharin [main effect of saccharin concentration: ($F_{(1,25)} = 11.950$, $P = 0.002$), main effect of drug: ($F_{(1,25)} = 0.830$, $P = 0.371$), concentration × drug interaction: ($F_{(1,25)} = 0.223$, $P = 0.641$), Fig. 2B] or in total volume of fluid consumed during the tastant study [main effect of saccharin concentration: ($F_{(1,25)} = 1.749$, $P = 0.198$), main effect of drug: ($F_{(1,25)} = 0.252$, $P = 0.620$), concentration × drug interaction: ($F_{(1,25)} = 0.081$, $P = 0.779$), Fig. 2C].

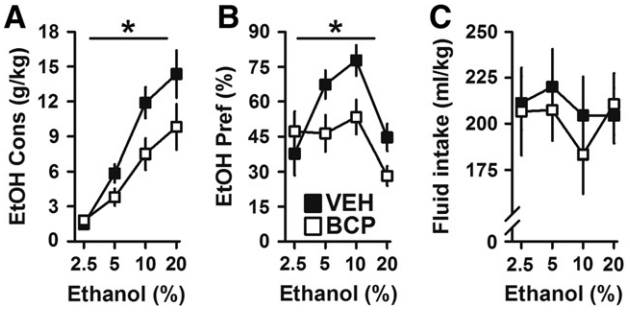

Fig. 1. Effect of BCP (50 mg/kg) on ethanol (2.5, 5, 10 and 20%) consumption and preference. A) Ethanol consumption calculated as grams of alcohol consumed per kilogram of body weight in male C57BL/6 mice. B) Ethanol preference expressed as ethanol consumed/total fluid consumed and C) Average total fluid (water + ethanol) intake. Data are expressed as mean ± SEM. * $P < 0.05$; vehicle (n = 13) and BCP (n = 14).

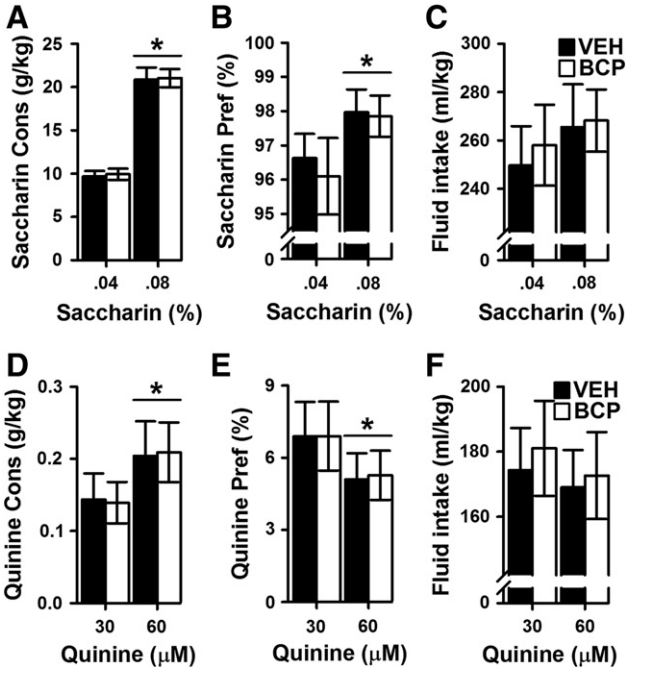

Fig. 2. Effect of BCP (50 mg/kg) on saccharin (0.04 and 0.08%) and quinine (30 and 60 μM) consumption and preference. A & D) Saccharin and quinine consumption calculated as grams of tastant consumed per kilogram of body weight in male C57BL/6 mice. B & E) Saccharin and quinine preference expressed as tastant consumed/total fluid consumed and C & F) Average total fluid (water + tastant) intake. Data are expressed as mean ± SEM. * $P < 0.05$; vehicle (n = 13) and BCP (n = 14).

Fig. 2 also shows quinine consumption (grams per kilogram) and preference for vehicle- and BCP-injected mice across two different quinine concentrations. A two-way ANOVA with repeated measures revealed a significant effect of quinine concentration for grams per kilogram quinine consumed ($F_{(1,25)} = 13.564$, $P = 0.001$). However, there was no effect of drug ($F_{(1,25)} = 0.004$, $P = 0.948$) or significant interaction of drug and quinine concentration ($F_{(1,25)} = 0.023$, $P = 0.880$) (Fig. 2D). A similar pattern was observed for quinine preference (Fig. 2E) [main effect of quinine concentration: ($F_{(1,25)} = 6.317$, $P = 0.019$), main effect of drug: ($F_{(1,25)} = 0.003$, $P = 0.954$), concentration × drug interaction: ($F_{(1,25)} = 0.002$, $P = 0.964$)]. Vehicle- and BCP-injected mice did not differ in their average total fluid intake [main effect of quinine concentration: ($F_{(1,25)} = 0.926$, $P = 0.345$), main effect of drug: ($F_{(1,25)} = 0.174$, $P = 0.681$), concentration × drug interaction: ($F_{(1,25)} = 0.352$, $P = 0.558$)] (Fig. 2F).

3.3. No tolerance to BCP was observed following chronic injection

In the experiments exploring the effects of BCP on alcohol, saccharin and quinine intake, the same mice were used in all experimental phases and the mice received daily BCP injections during all phases,

leading to a total of 32 BCP injections during the experiment. While chronic injections can be very useful for evaluation of the therapeutic potential of BCP, they can be pharmacologically challenging, though the long-term safety and efficacy showed that BCP is devoid of organ toxicities and genotoxicity, carcinogenicity as well as clastogenicity and mutagenicity (Molina-Jasso et al., 2009) and found useful in chronic debilitating pain (Klauke et al., 2014). To make sure that tolerance to BCP did not develop, so that the saccharin and quinine phases cannot be compared with the alcohol phase the same mice had access to two pipettes (water & 10% EtOH) while being injected with BCP (50 mg/kg, i.p.) during 4 consecutive days and ethanol intake and preference were measured as described in the Materials and methods section and results are depicted in Fig. 3. Two-way ANOVA indicated a main effect of treatment on ethanol consumption with BCP-injected mice displaying decreased ethanol intake compared to vehicle control mice ($F_{(1,25)} = 110.210$, $P \leq 0.001$) (Fig. 3A). Similarly, BCP decreased ethanol preference as indicated by a mean effect of treatment ($F_{(1,25)} = 217.721$, $P \leq 0.001$) (Fig. 3B). Finally and as expected BCP had no effect on total fluid intake ($F_{(1,25)} = 0.415$, $P = 0.525$) (Fig. 3C). At the end of BCP chronic injections, we analyzed body weight and the one-way ANOVA analysis indicated no main effect of treatment [vehicle: 29.65 ± 0.40 g; BCP: 29.16 ± 0.47 g] ($F_{(1,25)} = 0.590$, $P = 0.450$). Taken together, these results clearly indicated that tolerance to BCP did not develop and, therefore, the saccharin and quinine phases can be compared with the alcohol phase.

3.4. BCP dose-dependently lowered EtOH consumption and preference

In a two-bottle, free-choice paradigm in which the mice could drink either water or a 10% alcohol solution, the consumption and preference for EtOH were dose-dependently reduced in BCP-injected mice as shown in Fig. 4. One-way ANOVA with BCP treatment as the between-subjects factor revealed a main effect of dose ($F_{(3,48)} = 67.185$, $P \leq 0.001$). Post-hoc analysis showed that, compared to control, mice consumed similar amounts of EtOH when injected with 25 mg/kg BCP ($P = 1.000$). However, mice consumed significantly less EtOH when treated with 50 and 100 mg/kg BCP ($P \leq 0.001$ and $P \leq 0.001$, respectively) (Fig. 4A). Similarly, there was a main effect of dose on EtOH preference ($F_{(3,48)} = 170.362$, $P \leq 0.001$). Consequently, it seems that the mice preferred less EtOH when pretreated with 50 and 100 mg/kg BCP ($P \leq 0.001$ and $P \leq 0.001$, respectively) (Fig. 4B). In contrast, 25 mg/kg BCP had no effect on EtOH preference ($P = 1.000$). Finally, the average total liquid intake did not differ between the four groups indicating normal drinking behavior in BCP-treated mice ($F_{(3,48)} = 1.504$, $P = 0.226$) (Fig. 4C).

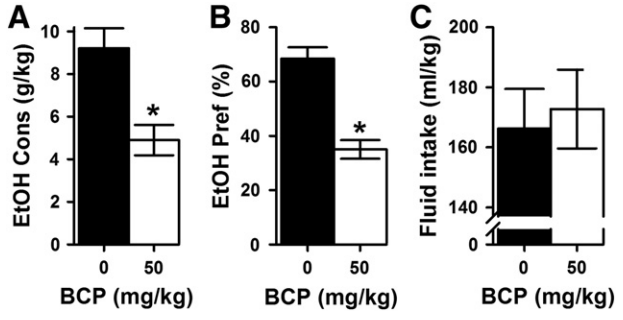

Fig. 3. No effect of BCP (50 mg/kg) tolerance on ethanol (10%) consumption and preference. A) Ethanol consumption calculated as grams of alcohol consumed per kilogram of body weight in male C57BL/6 mice. B) Ethanol preference expressed as ethanol consumed/total fluid consumed and C) Average total fluid (water + ethanol) intake. Data are expressed as mean ± SEM. * $P < 0.05$; vehicle (n = 13) and BCP (n = 14).

3.5. AM630 inhibited BCP-induced reduction of EtOH intake and preference

Whether BCP-reduced voluntary EtOH intake and preference could be abrogated by pretreatment with the CB_2-selective antagonist AM630, injected 15 min before BCP (50 mg/kg) was given (AM630–BCP; n = 7) was also tested. Control animals received daily injection of vehicle (DMSO–Oil; n = 7), BCP (DMSO–BCP; n = 8) or AM630 (AM630–Oil; n = 7). Based on previous studies, AM630 at 3 mg/kg was used (Asghari-Roodsari et al., 2010; Navarrete et al., 2013). The results showed that there was a main effect of drug treatment on EtOH consumption. In fact as depicted in Fig. 5A, the one-way ANOVA test with drug as the between-subjects factor revealed a significant effect of treatment on voluntary alcohol consumption ($F_{(3,25)} = 12.990$, $P \leq 0.001$). Post-hoc analysis revealed that BCP attenuated EtOH consumption ($P = 0.001$, DMSO–Oil *vs.* DMSO–BCP) as expected. This BCP-attenuated EtOH consumption was abrogated following AM630 pre-injection, evidenced by a significant difference between DMSO–BCP and AM630–BCP groups ($P = 0.003$). Interestingly, there was no significant difference between DMSO–Oil and AM630–BCP injected groups ($P = 1.000$). More importantly, AM630 did not influence EtOH consumption ($P = 0.702$, DMSO–Oil *vs.* AM630–Oil). Similarly, BCP-attenuated EtOH preference was also abrogated, when mice were pre-injected with the CB_2 receptor selective antagonist AM630. In fact, one-way ANOVA analysis revealed that there was a main effect of treatment on EtOH preference ($F_{(3,25)} = 44.165$, $P \leq 0.001$) (Fig. 5B). Post-hoc analysis indicated a significant difference between DMSO–Oil *vs.* DMSO–BCP ($P \leq 0.001$). Interestingly, the effect of BCP on EtOH preference was reversed with AM630 pre-injection ($P \leq 0.001$, DMSO–BCP *vs.* AM630–BCP). Also, there was no effect of AM630 alone on EtOH preference ($P = 0.434$, DMSO–Oil *vs.* AM630–Oil). Lastly, as depicted in Fig. 5C, combined AM630 and BCP administration had no effect on total fluid intake ($F_{(3,25)} = 0.529$, $P = 0.666$) which clearly demonstrated that the effect of the specific CB_2 receptor activation was the main cause for the change in alcohol consumption and preference.

3.6. BCP reduced EtOH-elicited CPP acquisition

The two-way ANOVA, repeated measure of the effect of BCP on the EtOH-CPP (Fig. 6) revealed a significant effect of the between-subjects factors: [main effect of conditioning: ($F_{(1,94)} = 132.697$, $P \leq 0.001$), main effect of treatment: ($F_{(3,94)} = 4.245$, $P = 0.007$)]. More importantly, the interaction between the two factors was significant ($F_{(3,94)} = 4.040$, $P = 0.009$). The ANOVA analysis of within-subject factors revealed that increased time was spent in the EtOH-paired compartment in the CPP test than in the pre-conditioning only in those groups receiving EtOH (main effect of time: $F_{(1,94)} = 110.647$, $P \leq 0.001$). Also, there was a significant interaction between time and drug treatment ($F_{(3,94)} = 3.170$, $P = 0.028$) as well as time and conditioning ($F_{(1,94)} = 112.504$, $P \leq 0.001$). Finally, the differences in time × treatment × conditioning interaction were also significant ($F_{(3,94)} = 4.357$, $P = 0.006$). Bonferroni's post-hoc analysis revealed that BCP treatment attenuated EtOH-CPP ($P = 0.040$, DMSO–Oil *vs.* DMSO–BCP) compared to vehicle. Importantly, BCP-inhibited EtOH-CPP acquisition was abrogated when mice were pre-injected with the CB_2 receptor antagonist, AM630 ($P = 0.019$, DMSO–BCP *vs.* AM630–BCP). Lastly, there was no significant difference between DMSO–Oil *vs.* AM630–Oil ($P = 1.000$) and no difference was found between DMSO–Oil *vs.* AM630–BCP ($P = 1.000$). Taken together, these data suggest that systemic injection of BCP induces a robust decrease in the conditioned-rewarding effects of EtOH and more importantly, these effects could be reversed with the CB_2 receptor antagonist, AM630.

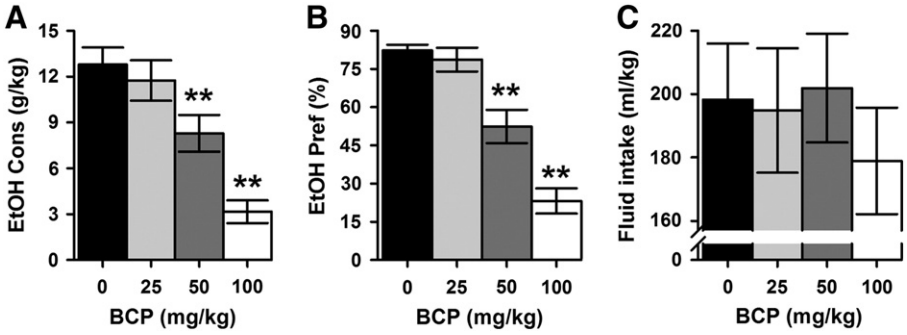

Fig. 4. Effect of BCP (0, 25, 50 and 100 mg/kg) on ethanol (10%) consumption and preference. A) Ethanol consumption calculated as grams of alcohol consumed per kilogram of body weight in male C57BL/6 mice. B) Ethanol preference expressed as ethanol consumed/total fluid consumed and C) Average total fluid (water + ethanol) intake. Data are expressed as mean ± SEM. ** $P < 0.001$; n = 13.

3.7. BCP exacerbated EtOH sensitivity in C57BL/6 mice

The effect of BCP on EtOH-induced loss of righting reflex (LORR) in C57BL/6 male mice was dependent on treatment ($F_{(3,72)} = 5.433$, $P = 0.002$) and dose of EtOH ($F_{(2,72)} = 91.516$, $P \leq 0.001$) with no significant treatment × dose interaction ($F_{(6,72)} = 0.372$, $P = 0.894$) (Fig. 7). Post-hoc analysis showed that injection of BCP increased the EtOH-induced hypnotic effect only at the lowest doses (3 and 3.4 g/kg) of EtOH tested. C57BL/6 male mice injected with BCP recovered from any motor coordination effect of EtOH at a significantly slower rate than the vehicle-treated group ($P = 0.016$, DMSO–Oil *vs.* DMSO–BCP). Most importantly, BCP-induced motor coordination was inhibited when mice were pre-injected with the CB_2 receptor antagonist, AM630 ($P = 0.002$, DMSO–BCP *vs.* AM630–BCP). Also, there was no significant difference between DMSO–Oil *vs.* AM630–Oil ($P = 1.000$) and no difference was found between DMSO–Oil *vs.* AM630–BCP ($P = 1.000$).

3.8. BCP had no effect on blood EtOH concentration

Finally, to test whether BCP utilized in this study was eliciting its effect by altering EtOH absorption and metabolism, the blood EtOH concentration (BEC) in vehicle- and BCP-injected mice was measured in the blood samples using an alcohol dehydrogenase assay. The animals were injected with either vehicle or BCP (50 mg/kg) 30 min before alcohol administration (3 g/kg). The BEC was determined by collecting blood 3 and 6 h after alcohol administration. The one-way ANOVA with repeated measure with drug as the between-subjects factor and time as the within-subject factor revealed a main effect of time ($F_{(1,14)} = 516.172$, $P \leq 0.001$). Therefore, in vehicle- and BCP-treated mice, average BECs decreased significantly over time and observed BECs were greater at 3 h [vehicle: 25.957 ± 0.294; BCP: 23.820 ± 0.694] than at 6 h [vehicle: 5.715 ± 0.669; BCP: 7.178 ± 1.529]. However, the rate of alcohol metabolism, as measured by the slope of the line, was not significantly different between the two groups ($F_{(1,14)} = 0.111$, $P = 0.743$). Therefore, the changes in alcohol sensitivity did not result from potential differences in the clearance of alcohol.

4. Discussion

The aim of this study was to examine the effects of the pharmacological modulation of CB_2 receptors using BCP (a naturally-available full-agonist of CB_2 receptors) on voluntary alcohol consumption and alcohol-elicited CPP. The results demonstrated for the first time that pharmacological activation of the CB_2 receptor using BCP is accompanied by a decrease in voluntary alcohol consumption and preference behaviors that cannot be attributed to differences in taste perception. Another major finding was that BCP-treated mice expressed less EtOH-CPP. Most importantly, the abolition of the effects of BCP in mice pretreated with AM630, a selective CB_2 receptor antagonist provided further evidence to support the fact that the effects of BCP are mediated by CB_2 receptor activation which has major involvement in the regulation of alcohol disorders.

In recent years, there has been accumulating evidence to suggest an interaction between alcohol dependence and the ECS including endocannabinoid ligands and cannabinoid receptors CB_1 and CB_2, which play an important and pervasive role in alcohol dependence (Basavarajappa, 2007; Onaivi, 2009). Therefore the targeting of CB_2 receptors would seem to be an interesting strategy for novel treatment of alcohol dependence knowing their existence in the CNS (Onaivi et al., 2006; Van Sickle et al., 2005; Xi et al., 2011) as well as their involvement in several neuropsychiatric disorders including depression, schizophrenia, anxiety and alcoholism (Le Foll et al., 2009; Moreira

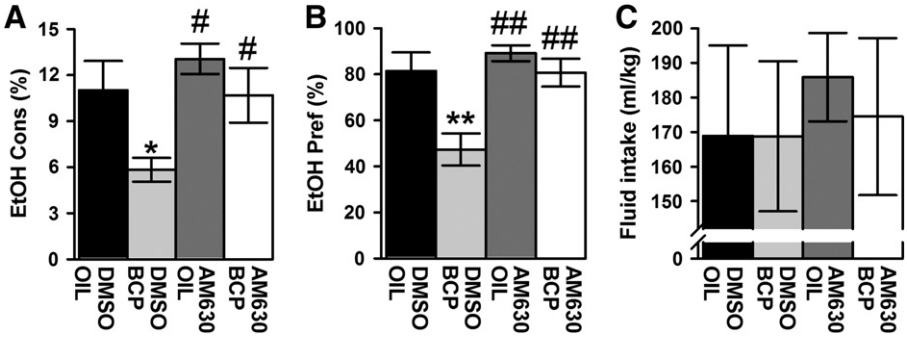

Fig. 5. Effect of vehicle, BCP (50 mg/kg), AM630 (3 mg/kg) and AM630–BCP on ethanol (10%) consumption and preference. A) Ethanol consumption calculated as grams of alcohol consumed per kilogram of body weight in male C57BL/6 mice. B) Ethanol preference expressed as ethanol consumed/total fluid consumed and C) Average total fluid (water + ethanol) intake. Data are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$ *vs.* DMSO–Oil. # $P < 0.05$, ## $P < 0.01$ *vs.* DMSO–BCP. DMSO–Oil (n = 7); DMSO–BCP (n = 8); AM630–Oil (n = 7) and AM630–BCP (n = 7).

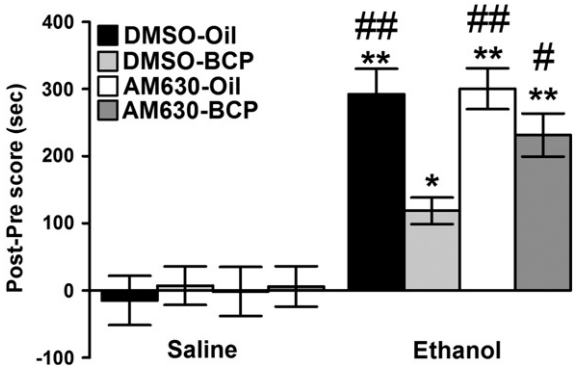

Fig. 6. Effect of vehicle, BCP (50 mg/kg), AM630 (3 mg/kg) and AM630–BCP on ethanol-elicited conditioned-place preference (CPP). Ethanol-elicited CPP scores are expressed as Post − Pre of time spent in the ethanol-paired chamber (total time of the test 900 s). C57BL/6 mice were conditioned with saline (left panel) or ethanol (1.5 g/kg; i.p.) (right panel). Before saline or ethanol conditioning, mice were previously injected with vehicle, BCP, AM630 or AM630–BCP. Data are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$ *vs.* Saline; # $P < 0.05$, ## $P < 0.01$ *vs.* DMSO–BCP. Saline conditioning: DMSO–Oil (n = 10); DMSO–BCP (n = 13); AM630–Oil (n = 12) and AM630–BCP (n = 12). Ethanol conditioning: DMSO–Oil (n = 11); DMSO–BCP (n = 15); AM630–Oil (n = 14) and AM630–BCP (n = 15).

et al., 2009; Moreira and Wotjak, 2010). There is certainly an emerging understanding that CB_2 receptor-activation is associated with vulnerability to alcohol consumption (Persidsky et al., 2011) and this is strongly supported by the results from this current study which showed that voluntary alcohol consumption and preference were significantly decreased in BCP-injected mice compared to the vehicle group. Recently, newly synthesized compounds which are analogues of CB_2 receptor agonists, have been shown to decrease alcohol self-administration without affecting total fluid intake (Vasiljevik et al., 2013). The results of the present study support these findings because BCP did not affect the total fluid intake and, unlike other agents used for alcohol addiction did not alter body weight during the treatment period, a favorable feature for its potential use in alcohol addiction. The results also supported those from another study in which the pharmacological activation of CB_2 receptors attenuated the effects of cocaine (Xi et al., 2011). The results from other studies using genetic models also provide much support to these conclusions. For example, mice developing EtOH preference showed reduced CB_2 receptor gene expression in the striatum and ventral midbrain (Onaivi et al., 2006) and deletion of the CB_2 gene has been demonstrated to increase preference and vulnerability for alcohol consumption (Ortega-Alvaro et al., 2013). The results from this current study are especially important because they are in direct agreement with those from previous studies which reported that CB_2 receptor activation decreased alcohol intake and preference [for example, see Ortega-Alvaro et al., 2013]. Therefore, this is the first report that pharmacological activation of the CB_2 receptor (using BCP) is accompanied by a decrease in voluntary EtOH consumption and preference behaviors which is not attributed to differences in the taste perception. More importantly, these effects of BCP were abrogated when mice were first injected with AM630, a selective CB_2 receptor antagonist. The CB_2 receptor activity difference in alcohol intake also cannot be associated with differences in total fluid intake between the two groups. Accordingly, other factors which are known to contribute to alcohol drinking behaviors were also examined. It is possible that the taste "palatability" of alcohol differs between vehicle- and BCP-injected mice. However, no differences in the consumption and preference of saccharin or quinine solutions were observed in the present study which indicates that there was no difference in the taste preference between the two groups.

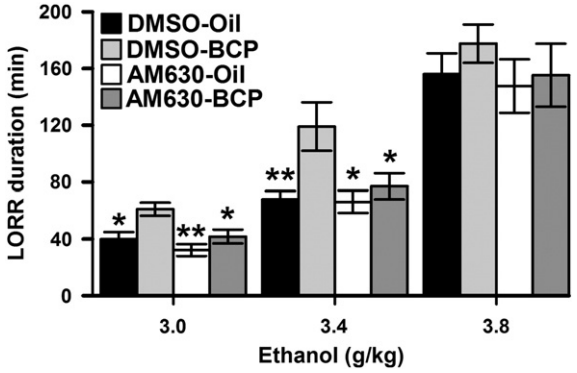

Fig. 7. Effect of vehicle, BCP (50 mg/kg), AM630 (3 mg/kg) and AM630–BCP on ethanol-induced loss of righting reflex (LORR). LORR was induced following injection of ethanol (3, 3.4 or 3.8 g/kg ethanol from 20% solution in isotonic saline). Before ethanol administration, mice were previously injected with vehicle, BCP, AM630 or AM630–BCP. Data are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$ *vs.* DMSO–BCP. DMSO–Oil (n = 7); DMSO–BCP (n = 7); AM630–Oil (n = 7) and AM630–BCP (n = 7).

A CPP test to measure the EtOH-induced conditioned reward in C57BL/6 mice was also performed. Although both groups developed EtOH-CPP, only vehicle-treated mice developed a reliable place-preference while BCP-injected mice showed almost no preference for the compartment paired with alcohol during conditioning. These observed favorable changes in CPP are very suggestive of the important role of CB_2 receptors in alcohol-rewarding properties. The results also showed that when given a choice of environment, the vehicle-injected mice preferred the alcohol-paired box. In contrast, the BCP-injected mice did not show any preference between the two boxes spending approximately equal time in each. The CPP results indicated that the CB_2 receptors were involved in the saliency of the environmental cues associated with alcohol as well as overall alcohol-induced drug-seeking behavior. It is possible that the reduced voluntary alcohol intake in BCP-injected mice might also reflect a decrease rather than an increase in alcohol reward. In addition to reduced consumption and preference of alcohol after BCP injection, the other major finding of the present study is that BCP-treated mice showed reduced EtOH-CPP acquisition.

To support the findings that the BCP has centrally-mediated effects and involves central CB_2 receptors, the alcohol pharmacodynamics in vehicle- and in BCP-treated mice were compared to assess whether the dissimilarities in voluntary alcohol consumption and preference could be due to a reduction of alcohol metabolism activity in BCP-injected mice. The results showed that there was no difference in the time-matched BEC between the two groups, suggesting no differences in alcohol metabolism levels. These results further support the concept of a role for central CB_2 receptors in alcohol consumption and preference and substantiate the main outcome of the current study being that CB_2 receptor activation is associated with attenuated voluntary alcohol consumption and preference in mice.

Recently, it has also been reported that the endocannabinoid mechanism of action depends not just on cannabinoid receptor activation. In fact, endocannabinoid-sensitive non-cannabinoid receptors may also mediate endocannabinoid action (O'Sullivan and Kendall, 2010). BCP has been shown to activate CB_2 which has been proposed to trigger the nuclear receptors, peroxisome proliferator-activated receptor-gamma (PPAR-γ) (Bento et al., 2011). Recently, Stopponi and colleagues showed that thiazolidinedione (TZD) drugs such as pioglitazone and rosiglitazone decrease alcohol consumption and preference in rats and that this effect was blocked by a PPAR-γ antagonist (Stopponi et al., 2011), indicating that it is mediated by PPAR-γ receptors in the brain. In addition, PPAR-γ activation has also altered alcohol's rewarding properties, prevented relapse to alcohol abuse, potentiated the effects of naltrexone on alcohol drinking and relapse and did not affect food or saccharine self-administration as well as the alcohol or glucose metabolism (Stopponi et al., 2011). Given the CB_2 receptors (Onaivi, 2006) and PPAR-γ co-exist in the CNS (Moreno et al., 2004; Sarruf et al., 2009), the cannabinoid-related molecules which activate CB_2 and PPAR-γ, may both become good candidates for the discovery of

novel drugs to treat alcohol disorders. In a previous study, BCP did not show NMDA, GABAergic, glutaminergic and serotonergic receptor-mediated activity and suggested that anxiolytic activity was due to some other mechanism (Bento et al., 2011; Galdino et al., 2012).

In summary, the results from the present study clearly demonstrated that BCP significantly reduced voluntary alcohol intake and CPP scores of mice through CB_2 receptor activation which is further substantiated by a significant reversal of the effects of BCP by CB_2 receptor antagonist, AM630. The CB_2 receptor mediated effects of BCP on alcohol consumption, preference and reward properties may offer clues to the pathogenesis of alcoholism and open additional avenues for potential therapeutic strategies. Furthermore, the results from this study also suggest that the BCP-like compounds could be promising for use in the treatment of alcohol addiction disorders.

Role of the funding source

The research grant supports from the United Arab Emirates University and the National Research Foundation, UAE to AB (Grant No. 31M082) and SO (Grant No. 31M099) are duly acknowledged. The funders had no further role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Author's contribution

AB was responsible for the study concept and design. SAM, SO, EAM, MAA, and AB contributed to the acquisition of animal data. AB performed the data analysis and SAM, MAA and EAM helped in the interpretation of findings. AB, MAA and SO drafted the manuscript. All the authors provided critical revision of the manuscript for important intellectual content and reviewed content and approved final version for publication.

Acknowledgments

The authors would like to acknowledge Mr. Mohamed Elwasila and Mr. Mohamed Shafiullah for their technical assistance and Dr. Mahmoud Hag Ali from the Animal Research Facility for his suggestions on animal care and welfare. The authors would also like to thank Professor Keith M. Bagnall for his critical and careful proofreading.

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