Published in final edited form as:

Psychopharmacology (Berl). 2017 October ; 234(19): 2955–2970. doi:10.1007/s00213-017-4691-9.

Blockade of alcohol escalation and “relapse” drinking by pharmacological FAAH inhibition in male and female C57BL/6J mice

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Abstract

Background—Anandamide (AEA)-dependent signaling is regulated by the catabolic enzyme fatty acid amide hydrolase (FAAH). Several lines of evidence have demonstrated that FAAH and AEA are involved in the behavioral effects of alcohol. Therefore, we investigated whether a selective FAAH inhibitor, URB597 (Cyclohexylcarbamic acid 3'-[aminocarbonyl]-[1,1'-biphenyl]-3-yl ester), altered alcohol intake in mice in a voluntary alcohol drinking model.

Methods—Mice, subjected to 3 weeks of chronic intermittent access (IA) in a two-bottle choice paradigm with 24-h access every other day, developed rapid escalation of alcohol intake and high preference. We evaluated the pharmacological effects of URB597 after both acute (1-day) withdrawal from chronic IA and 1-week withdrawal using the alcohol deprivation effect (ADE) model. AEA and N-acyl ethanolamide (NAE) abundances were determined after chronic IA, acute (1-day) or long-term (1 and 2 weeks) withdrawal in four brain regions.

Results—Acute pretreatment with URB597 reduced alcohol intake and preference after acute withdrawal. This effect was blocked by pretreatment with a selective type 1 cannabinoid receptor (CB1) antagonist, suggesting a CB1-mediated mechanism. Both single- and multiple- dosing regimens with an effective dose of URB597 prevented the ADE, with no tolerance development after the multi-dosing regimen. AEA and NAE levels were transiently increased in all brain regions measured after acute withdrawal, indicating that the endocannabinoid system is involved in acute alcohol withdrawal stress response.

Conclusion—FAAH inhibitors reduce alcohol escalation and “relapse” drinking in mice.

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Conflict of interest: All authors declare that they have no conflicts of interest.

Contributors: YZ designed the study, conducted behavioral studies, wrote the protocol, managed the literature searches and analyses, undertook the statistical analysis, and wrote the manuscript; BIS, SSG designed and conducted the experiments on tissue eCB measurements; JG, FSL designed and conducted the experiments on FAAH Western blot; MJK contributed to the final versions of manuscript writing; and all have approved the final manuscript. Special thanks to Austin Kordic for providing the comments and corrections on the manuscript.

Keywords

FAAH inhibitor; URB597; anandamide; N-acyl ethanolamide; alcohol escalation drinking; alcohol deprivation effect

Introduction

The endocannabinoid (eCB) system consists of cannabinoid receptors (CB1 and CB2) and endogenous cannabinoids (including anandamide [AEA] and 2-arachidonoyl glycerol). Pharmacological blockade of CB1 receptors reduces alcohol drinking in mice [Arnone et al. 1997], decreases alcohol intake and the motivation to consume alcohol [Colombo et al. 1998; Gallate and McGregor 1999] and blocks alcohol seeking in rats [McGregor et al. 2005], suggesting that the eCB/CB1 system is involved in the positive reinforcing properties and consumption of alcohol. In addition, mice lacking CB1 have reduced alcohol drinking or preference [Hungund et al. 2003; Wang et al. 2003; Naassila et al. 2004], and alcohol-induced reward [Houchi et al. 2005]. Together, the increased eCB/CB1 activity is likely to promote alcohol intake during early stages of alcohol drinking by potentiating acute alcohol reward [Manzanares et al. 1999]. In apparent contrast, eCB/CB1 signaling deficiency occurs after chronic alcohol exposure and protracted withdrawal, which may promote alcohol intake through a negative reinforcement mechanism. Several findings support this notion: [1] down-regulation of CB1 expression and function is observed during protracted alcohol withdrawal [Mitrirattanakul et al. 2007; Varodayan et al. 2016]; [2] in humans, *in vivo* imaging studies find decreased CB1 availability in heavy-drinking alcoholics that persists into abstinence [Hirvonen et al. 2013; Ceccarini et al. 2014]; and [3] alcohol dependent patients have lowered AEA levels in plasma during recent abstinence [Mangieri et al. 2009].

The AEA-dependent signaling has been shown to be regulated by the enzyme involved in its catabolism, fatty acid amide hydrolase (FAAH) [Cravatt et al. 1996, 2001]. Several lines of evidence have demonstrated that AEA is involved in the behavioral effects of alcohol: inhibition or genetic deletion of FAAH, with resultant increases in AEA levels [Cravatt et al. 2001], increases alcohol consumption and preference [Basavarajappa et al. 2006; Blednov et al. 2007]. The FAAH C385A polymorphism (with increased eCB activity due to impaired FAAH function) has been reported to be associated with increased alcohol abuse and dependency [e.g., Sipe et al. 2002; Sloan et al. 2017], and consistently, we found that the knock-in mice with the human FAAH C385A polymorphism had increased alcohol consumption [Zhou et al. 2016], with decreased anxiety-like behavior [Dincheva et al. 2015].

Withdrawal from alcohol is associated with increased stress responsivity and persistent negative affective symptoms, such as anxiety and depression, the severity of which are closely associated with alcohol relapse susceptibility [Koob & Kreek 2007]. eCBs exert prominent modulatory influence on the extended amygdala and corticostriatal circuits, and stress exposure disrupts eCB-enriched regions that participate in emotional control [Serrano et al. 2012; Dincheva et al. 2015; Morena et al. 2016]. Specifically, the relatively deficient eCB function is associated with increased anxiety and depression, and as such, impaired

eCB activity may contribute to the negative affective states and increased stress responsivity that underlie negative reinforcement mechanisms driving alcohol drinking by dependent individuals and that contribute to alcohol relapse following periods of abstinence [Parsons & Hurd, 2015]. Pharmacologic and genetic manipulations (knockout or knock-in) of FAAH have been implicated in anxiolytic and anti-depressive behaviors [Bortolato et al. 2007; Gunduz-Cinar et al. 2013; Kathuria et al. 2003; Moreira et al. 2008; Carnevali et al. 2015]. However, there is no study on FAAH inhibitors in rodent models with alcohol withdrawal from high intake, though FAAH inhibition decreases anxiety-like behaviors that are present during alcohol withdrawal [Cippitelli et al. 2008].

Our hypothesis is that enhanced eCB signaling by FAAH inhibition opposes the effect of alcohol withdrawal driving alcohol escalation and “relapse” drinking. In this study, therefore, we first investigated whether a selective FAAH inhibitor URB597 alters voluntary alcohol drinking in mice during acute (1 day) withdrawal from chronic (3-week) intermittent access (IA) alcohol drinking, to explore its potential for development as a therapeutic agent for alcoholism. In the IA model, the mice had access to alcohol drinking for 3 weeks in a two-bottle choice paradigm with alcohol drinking every other day, and the mice developed high alcohol consumption (15–25 g/kg/day). Thus, chronic IA mice constitute an appropriate animal model for studying voluntary alcohol drinking, as they display rapid escalation of high alcohol intakes [Hwa et al. 2011] and develop the alcohol deprivation effect (ADE, modeling the relapse episodes that occur in human alcoholics) [Zhou et al. 2017]. Consequently, we determined the pharmacological effects of URB597 in an ADE model, which allows for access to alcohol after 1 week of withdrawal. For comparison, we compared the effect of URB597 in the drinking-in-the-dark (DID) model, which allows for limited access (4 h/day) with low intake (<5–6 g/kg/day) [Rhodes et al. 2005], and models “binge” drinking to the point of intoxication. Finally, we assessed the eCB system at a biochemical level in the IA model, by measuring relative abundances of AEA, other N-acyl ethanolamide (NAE) lipids and of FAAH protein after chronic IA and acute (1 day) or long-term (1 and 2 weeks) withdrawal, providing a dynamic profile of the eCB system in response to chronic alcohol IA consumption and subsequent withdrawal.

MATERIAL AND METHODS

1. Animals

Male adult C57BL/6J (B6) mice (8 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in a temperature-controlled room (21 °C). Mice were placed on a 12-hour reverse light-dark cycle (lights off at 7:00 am) upon arrival, and acclimated for a week prior to testing. During the first day of testing (i.e., session 1), all mice were 9 weeks of age. Mice were individually housed in ventilated cages fitted with steel lids and filter tops and given *ad libitum* access to food and water. Animal care and experimental procedures were conducted according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources Commission on Life Sciences 1996), and were approved by the Institutional Animal Care and Use Committee of the Rockefeller University.

2. Materials

FAAH inhibitor URB597 (Cyclohexylcarbamic acid 3'-[aminocarbonyl]-[1,1'-biphenyl]-3-yl ester) and selective CB1 antagonist AM251 [Tocris Inc., Bristol, UK] were dissolved in 1% DMSO plus physiological saline and in 5% Cremophor in physiological saline, respectively. Alcohol solutions (7.5%, 15% and 30% v/v) were prepared from 190 proof absolute ethyl alcohol (Pharmco-AAPER, Brookfield, CT, USA) and dissolved in tap water. Sucrose and saccharin [Sigma-Aldrich Inc., St. Louis, MO, USA] were diluted in tap water. Optima liquid chromatography-mass spectrometry (LC/MS) grade acetonitrile [Fisher Scientific, Pittsburgh, PA, USA] was used for metabolite extractions and liquid chromatography. Deuterated anandamide standard was purchased from Cayman Chemical [Ann Arbor, Michigan, USA].

3. Procedures

3.1 Chronic Intermittent Access (IA)—This model of alcohol drinking in C57BL/6J mice has been widely used for several years by many laboratories [e.g., Hwa et al. 2011; Zhou et al. 2017] (Table 1A).

3.1.1. The 3-week IA model: Mice had access to alcohol drinking in their home cages for 3 weeks. Food and water were available at all times in this two-bottle choice paradigm with chronic alcohol exposure every other day. This IA model was similar to earlier protocols [e.g., Hwa et al. 2011], with our modifications [Zhou et al. 2017]: At the time when the mice started individual housing (1 week before the experiments), the original water bottles were exchanged for those with sipper tubes to acclimate the mice to the sipper tubes (without ball bearings). Starting at 3 hours after lights off (10:00 am), both the water and alcohol (7.5%, 15% or 30%) solution sipper tubes were placed on their home cages. These sipper tubes were 10-ml pipettes that were cut at both ends, sealed with a rubber stopper, and fitted with a stainless steel straight sipper tubing. The sipper tubing contained a ball bearing at the end to prevent alcohol leakage. The left/right position of the tubes was randomly set to avoid the development of side preference. The alcohol pipettes were refilled with fresh alcohol solution, and left for 24 hours before being replaced with the water tubes. Alcohol solutions were prepared fresh every 48 hours by mixing alcohol with tap water to reach the appropriate (v/v) alcohol concentration. Body weights were recorded every 2 days. Alcohol and water intake values were recorded after 4, 8 and 24 hours of alcohol access in the drinking days (to the nearest 0.1 ml). These data were used to calculate consumed alcohol intake (i.e., g/kg) and relative preference for alcohol (i.e., alcohol intake/total fluid intake).

Mice exposed to the 2-bottle “alcohol vs. water” choice regimen every other day rapidly acquired alcohol drinking behaviour, and subsequently daily alcohol intake rose to levels averaging approximately 15–20 g/kg/day after 3 weeks with 15% alcohol, with a high preference ratio above 0.8–0.9. This 3-week voluntary IA alcohol exposure was found to give rise to blood alcohol concentrations (BAC) around 0.50 – 0.63 mg/ml, which were within the range required to produce specific psycho-pharmacological effects [Hwa et al. 2011; Zhou et al. 2017].

The mice randomized as the vehicle-treated and drug-treated groups had matched body weight and similar alcohol intake 24 hours before the test day. The drug dissolved in vehicle was administered by an experimenter, blinded to the treatments given to the experimental groups.

3.1.2. Single, acute administration in the 3-week IA model: After 1 day of withdrawal, alcohol (7.5%, 15% or 30% concentrations) was presented 30 min after a single injection of URB597 (0.125, 0.25, 0.5, or 1 mg/kg in 1% DMSO, i.p.) or vehicle, and then alcohol and water intake values were recorded (Table 1A). The URB597 doses chosen (0.125–1 mg/kg) were based on the literature [Blednov et al. 2007; Manwell et al. 2009].

In this experiment, we also tested the effect of URB597 at 0.5 mg/kg after 1 day of withdrawal in female mice exposed to 3 weeks of IA. We did not observe any sex differences in the effects of URB597 after either single or repeated administration in the following experiments, suggesting that the estrous cycle and associated hormones might not be important factors in the response to these treatments in females.

Lastly, we used selective CB1R antagonist AM251 to pharmacologically block the CB1R to confirm that the URB597 effects were mediated via CB1R. Mice were pretreated with AM251 (0.1 mg/kg) in 5% Cremophor plus saline (i.p.) 30 min before one URB597 (0.5 mg/kg) or vehicle (1% DMSO) injection 30 min before the drinking test. The AM251 dose chosen (0.1 mg/kg) was based on our recent study [Zhou et al. 2016].

3.2 Chronic (3-week) drinking-in-the-dark (DID) (Table 1B)—This model of alcohol drinking in C57BL/6J mice has been widely in use by many laboratories [e.g., Rhodes et al. 2005]. In this one-bottle paradigm, alcohol exposure was every day, and alcohol drinking was measured during 4 hours of alcohol access. This chronic DID model was similar to earlier protocols [Rhodes et al. 2005], with our modifications [Zhou et al. 2017]; The procedures were identical to the above IA with the following exceptions: Starting at 3 hours after lights off (10:00 am), the water bottle was replaced with one 10-ml alcohol (15%) pipette, and left for 4 hours before being replaced with the water bottle. Alcohol intake values were recorded after 4 hours of alcohol access every day (to the nearest 0.1 ml). These data were used to calculate alcohol intake (i.e., g/kg). After 3 weeks of DID, the mice assigned to the vehicle-treated and URB597-treated groups with matched body weight had similar 15% alcohol intake 1 day before the test day. The URB597 doses (0.25 and 0.5 mg/kg) were chosen based on the results of the above IA alcohol study. On the test day (20 hours of withdrawal), alcohol (15% concentration) was presented 30 min after a single injection of URB597 or vehicle. Control groups: mice experienced DID for 3 weeks, and received one vehicle injection (1% DMSO, i.p.) before the 4-h drinking test. Test groups: mice experienced DID for 3 weeks, and received one URB597 injection (0.25 or 0.5 mg/kg in 1% DMSO, i.p.) before the test.

3.3. Sucrose (caloric reinforcer) and saccharin (non-caloric reinforcer) drinking—With 0.5 mg/kg URB597 (the most effective dose tested for reducing alcohol intake), we further tested the specificity of the action of URB597 on alcohol intake using sucrose or saccharin drinking behavior after single, acute administration in alcohol naïve

mice or following the IA. The URB597 dose was chosen also based on the literature for sucrose drinking tests (0.1–1 mg/kg) [Bortolato et al. 2007; Rademacher & Hillard, 2007]. The sucrose or saccharin preference test in mice is sensitive to the function of brain rewarding systems and is widely used to measure the expression of anhedonia after chronic stress [Lim et al. 2012] and during alcohol abstinence [Pang et al. 2013]. In the following experiments, 15% alcohol IA exposure was identical to those in the above experiment as described in section 3.1.1. After 3 weeks of IA, the alcohol tube was switched to sucrose for 3 sessions with stable intakes. The mice assigned to the vehicle-treated or URB597-treated groups had similar sucrose intake 24 hours before the test day. On the test day (after 8 days of alcohol withdrawal), sucrose (2% or 4%) and water intake values were recorded after 4, 8 and 24 hours of sucrose access. In parallel separate experiments, saccharin drinking (0.1% or 0.2%) was tested after 3 weeks of IA with an identical procedure.

3.3.1. Single, acute administration of URB597 on sucrose or saccharin drinking after 3-week IA: A single i.p. injection of URB597 (0.5 mg/kg) in 1% DMSO or vehicle was given 30 min before the sucrose and saccharin were presented. Mice were assigned to one of two treatment groups: vehicle as control and URB597.

3.3.2. Single, acute administration of URB597 on saccharin drinking in alcohol-naïve mice: The procedures were identical to the above, except the mice were exposed to saccharin or sucrose only (after 3 training sessions).

3.4. The alcohol deprivation effect (ADE) after 1-week withdrawal from 3-week IA (Table 1C)—Similar to the above, mice were given access to 15% alcohol and water for 24 h, and then received 24-h access to alcohol on alternating days for 3 weeks. In the baseline session, 15% alcohol and water intake values were recorded at 4, 8 and 24 hours after 3 weeks of IA. The alcohol bottles were then removed from the cages leaving the mice with access to food and water *ad libitum*. After 7 days of withdrawal, the alcohol (30%) bottles were presented to the mice 3 hours after the dark cycle and the alcohol and water intake values were recorded at 4, 8 and 24 hours in the ADE session. In the following two experiments, we also tested the effect of URB597 at 0.5 mg/kg in female mice with the ADE model.

3.4.1. Single, acute administration in the ADE model: The mice assigned to the vehicle-treated or URB597-treated groups had similar alcohol intake in the baseline session. Control groups: mice received one vehicle injection (1% DMSO, i.p.) before the ADE test after 1-week withdrawal; and Test groups: mice received one URB597 injection (0.5 mg/kg in 1% DMSO, i.p.) before the ADE test after 1-week withdrawal. Then, alcohol was presented 30 min after URB597 or vehicle injection, and then alcohol and water intake values were recorded after 4, 8 and 24 hours of alcohol access. The URB597 dose chosen (0.5 mg/kg) was based on the above IA experiments.

3.4.2. Repeated administration in the ADE model: In the following experiments, alcohol exposure procedure was identical to the above experiment in section 3.4.1, with the following exceptions: mice received 5 consecutive administrations of vehicle or URB597 (0.5 mg/kg) during the 1-week withdrawal. On the ADE test day, alcohol (15%) was

presented 1 day after the last URB597 injection, and then alcohol and water intake values were recorded after 4, 8 and 24 hours of alcohol access.

3.5. N-Acyl Ethanolamide (NAE) extraction for mass spectrometry (MS) quantification

3.5.1. Brain tissue collection and extraction: Alcohol exposed and control (water) male mice from the 3-week chronic IA model were sacrificed by decapitation in the following groups: No alcohol withdrawal (immediately following the last day of alcohol exposure), 1-day withdrawal, 1-week withdrawal, and 2-week withdrawal. Brain regions (nucleus accumbens [NAc], prefrontal cortex [PFC], basolateral amygdala [BLA] and cerebellum) were dissected and frozen on dry ice within 1 minute of sacrifice. Brain regions from two mice were combined for each sample to ensure sufficient sample size.

NAEs were extracted from brain tissue using a protocol adapted from a previously described method [Patel et al. 2003]: Stainless steel bead-beating beads were added to the Eppendorf tubes containing brain tissue. 400µL of acetonitrile (ACN) was added to the samples. 1µL of 1ng/µL deuterated anandamide ([²H₈]AEA) was added as an internal standard (IS). Bead beating homogenization was performed using a TissueLyser II bead beater (Qiagen) for 1 minute at 30/s frequency. Samples were maintained on dry ice until homogenization, after which they were kept on ice. Homogenates were centrifuged in a table top centrifuge cooled to 4°C at maximum speed for 10 min to pellet brain tissue. The supernatant was removed to a new Eppendorf and brain tissue was homogenized twice more in 400µL ACN to ensure complete extraction of NAEs. Supernatants were combined and stored overnight at -20°C to precipitate particulates. Remaining brain tissue protein pellets were stored at -80°C until protein quantification. Brain extracts were centrifuged at max speed for 10 min and supernatants were removed to a new Eppendorf. Extracts were dried under vacuum in a vacufuge, reconstituted in 500 µL ACN to remove metabolites from the side of the tube and dried again. Dried extracts were stored at -80°C until reconstitution for mass spectrometry.

3.5.2. Protein quantification: Remaining protein pellets from brain tissue homogenization were dried under vacuum in a vacufuge and dissolved in 0.2N NaOH at 95°C and protein content was determined using standard Bio-Rad Bradford protein quantification protocol.

3.5.3. Liquid chromatography-mass spectrometry (LC-MS) NAE quantification: Dried brain extracts were reconstituted to the equivalent of 1µL 70% ACN in water per 100µg protein to correct for differences in brain tissue size. The relative abundances of NAEs were determined by liquid chromatography-electrospray ionization mass spectrometry using a 1290 Infinity LC system (Agilent Technologies) coupled to a 6460 Triple Quadrupole Mass Spectrometer (Agilent Technologies). Metabolites were separated on a ZORBAX SB-Aq Rapid Resolution reversed-phase column (2.1 × 100mm, 1.8 micron) using mobile phase A (deionized water, 1% ACN, 1mM ammonium formate, 0.1% formic acid) and mobile phase B (ACN, 1% deionized water, 1mM ammonium formate, 0.1% formic acid). Samples were eluted using the following gradient:

Time (min)	A [%]	B [%]	Flow [mL/min]
0	65	35	0.4
0.5	65	35	0.4
0.6	45	55	0.4
10	45	55	0.4
10.1	0	100	1
13.1	0	100	1
13.2	65	35	0.4
16.2	65	35	0.4

NAEs were detected in positive ion mode. Standard multiple reaction monitoring methodology was employed for detection of anandamide (m/z 348.3 $\rightarrow$ 62.1; retention time = 4.8 min), deuterated anandamide (m/z 356.3 $\rightarrow$ 62.1; retention time = 4.8 min), palmitoyl ethanolamide (m/z 300.3 $\rightarrow$ 62.1; retention time = 5.5 min) and oleoyl ethanolamide (m/z 326.3 $\rightarrow$ 62.1; retention time = 6.2 min).

3.5.4. MS data analysis: MS data was extracted and integrated using Agilent Technologies Masshunter Quantitative Analysis software. Raw ion count abundances were then normalized as follows: internal standard ($[^2\text{H}_8]\text{AEA}$) abundances were normalized by multiplying by $\frac{\text{sample protein abundance}}{\text{average protein abundance}}$ to correct for different reconstitution volumes. These normalized IS abundances were then used to normalize the raw abundances by multiplying by $\frac{\text{average } [^2\text{H}_8]\text{AEA abundance}}{\text{sample } [^2\text{H}_8]\text{AEA abundance}}$ to correct for handling error. $[^2\text{H}_8]\text{AEA}$ was used as the IS for all NAEs $^2\text{H}_8$ AEA abundance with the assumption that physical properties of the compounds are similar.

3.6. Western Blot—Alcohol exposed and control (water) male mice from the 3-week chronic IA model were sacrificed by decapitation in the following groups: water control, 1-day withdrawal, 1-week withdrawal, and 2-week withdrawal. Prefrontal cortex was dissected and frozen on dry ice within 1 minute of sacrifice, and the tissue from two mice were combined for each sample to ensure sufficient sample size. Tissue lysates were prepared in lysis buffer containing 1% NP-40, 1% Triton, 10% Glycerol, 1% protease and phosphatase inhibitors (Calbiochem, San Diego, USA) in 1x buffered saline on ice. Samples were triturated and spun down at 12,000 rpm for 10 min at 4°C. Supernatants were collected and protein concentration measured by Bradford (Bio-Rad, Hercules, USA). Proteins were resolved by SDS–PAGE electrophoresis using ready NuPAGE 10% Bis-Tris gel (Invitrogen, Carlsbad, USA) and probed as previously described (Dincheva et al. 2015) using anti-FAAH (1:500). Secondary HRP-conjugated antibodies were used at 1:3000 (Santa Cruz, Dallas, USA).

4. Data analysis

We performed power analyses to determine the number of mice required to provide statistically significant results, based on the levels of differences seen previously [Zhou et al.

2016, 2017], and predicted that these studies require 6–8 animals per group. In the experiments with URB597, alcohol intake, water intake, total fluid and preference ratio differences between the different groups were analyzed using two-way ANOVA with repeat measures for treatment (vehicle vs drug) and for time (0–4h, 5–8h, vs 9–24h interval) in each sex (Figure 1). For dose response analysis on URB597, group differences for alcohol intake and preference ratios at the 4-hour recording time were analyzed using one-way ANOVA for treatments with different doses (Figure 2). In the experiments with URB597 with 7.5 and 30% alcohol, alcohol intake and preference ratio differences across the different groups were analyzed using two-way ANOVA for treatment (vehicle vs drug) and for concentration (7.5% vs 30%) (Table 2). In the experiments with URB597 combination with AM251, alcohol intake and preference ratio differences across the different groups were analyzed using one-way ANOVA for different treatments (Table 3). In the experiments with URB597 with 0.2 and 0.4% saccharin, saccharin intake and preference ratio differences across the different groups were analyzed using two-way ANOVA for treatment (vehicle vs drug) and for concentration (0.2% vs 0.4%) (Figure 3). In the ADE experiments, alcohol intake differences across the different groups were analyzed using two-way ANOVA for treatment (vehicle vs drug) and for session (baseline vs ADE) in each sex, with testing our *a priori* hypothesis that there was an ADE based on the published findings [e.g., Vengeliene et al. 2014; Zhou et al. 2017] (Figures 4, 5). For NAE quantification, abundance differences between water and alcohol groups were analyzed using a two-tailed Student's t-test followed by Bonferroni *post-hoc* tests (Figure 6) or one-way ANOVA with different time-points (Figure 7). All the ANOVA analysis was followed by Newman-Keuls *post-hoc* tests. The accepted level of significance for all tests was $p < 0.05$. All statistical analyses were performed using *Statistica* (version 5.5, StatSoft Inc, Tulsa, OK).

RESULTS

1. Single, acute administration of URB597 reduced alcohol (but not sucrose) intake and preference after 1 day of withdrawal from chronic IA

1.1. Single, acute URB597 at 0.5 mg/kg reduced 15% alcohol intake and preference in male and female mice—In male mice, for intake, two-way ANOVA with repeat measure revealed a significant effect of URB597 treatment [$F(1, 13) = 4.6, p < 0.05$], and *post hoc* analysis showed that the URB597-treated males had less intake than the vehicle-treated ones at 4 hours [$p < 0.01$] (Figure 1A). In comparison to the vehicle controls, 0.5 mg/kg URB597 reduced mean alcohol intake by 40–60% after 4 hours, associated with a compensatory increase in water intake (resulting in virtually unchanged total fluid intake [Table S1]). For preference ratio at this dose, two-way ANOVA with repeat measure revealed a significant interaction between URB597 treatment and time interval [$F(1, 26) = 6.2, p < 0.01$], and *post hoc* analysis showed that the URB597-treated males had less preference than the vehicle-treated ones at 4 hours [$p < 0.05$] (Figure 1B).

In this experiment, we also tested the effect of URB597 at 0.5 mg/kg in female mice after 3 weeks of IA, and URB597 had a similar reducing effect on alcohol intake and preference. As no significant sex difference in the effects of URB597 was found (Table S2), the female data are presented separately (Figures 1C, 1D). For intake, two-way ANOVA with repeat

measure revealed a significant effect of URB597 treatment [$F(1, 14) = 4.8, p < 0.05$], and *post hoc* analysis showed that the URB597-treated females had less intake than the vehicle-treated ones at 4 hours [$p < 0.05$] (Figure 1C). For preference ratio, two-way ANOVA with repeat measure revealed a significant effect of URB597 treatment [$F(1, 14) = 8.6, p < 0.05$], and the URB597-treated females had less preference than the vehicle-treated ones at both 0–4 and 5–8 hour intervals [$p < 0.01$ for both, *post hoc* analysis] (Figure 1D).

1.2. Dose-response of URB597 in male mice—At the 4-hour time point, the full-dose response of single, acute URB597 administration (0, 0.125, 0.25, 0.5 and 1 mg/kg) in terms of 15% alcohol intake and preference is presented in Figure 2. For intake (Figure 2A), one-way ANOVA revealed a significant effect of URB597 [$F(4, 28) = 9.6, p < 0.001$], and *post hoc* analysis showed that: (1) in comparison with the vehicle group, the URB597-treated males had less intake than the vehicle-treated ones at 0.25, 0.5 and 1.0 mg/kg doses [$p < 0.05, p < 0.01$ and $p < 0.05$, respectively]; and (2) the reductions at 0.5 mg/kg was greater than the one at 0.125 mg/kg [$p < 0.05$]. For preference ratio (Figure 2B), one-way ANOVA revealed a significant effect of URB597 [$F(4, 28) = 5.4, p < 0.005$], and *post hoc* analysis showed that (1) in comparison with the vehicle group, the URB597-treated males had less preference than the vehicle-treated ones at both 0.5 and 1.0 mg/kg doses [$p < 0.01$ and $p < 0.05$, respectively]; and (2) the reduction at 0.5 mg/kg was greater than the one at 0.125 mg/kg and 0.25 mg/kg [$p < 0.05$ for both].

1.3. Single, acute URB597 (0.5 mg/kg) reduced 30% (but not 7.5%) alcohol intake and preference in male mice—For alcohol intake after 4 hours (Table 2), two-way ANOVA revealed a significant effect of URB597 treatment [$F(1, 20) = 7.5, p < 0.05$], alcohol concentration [$F(1, 20) = 14.6, p < 0.005$], and a significant interaction between URB597 and alcohol concentration [$F(1, 20) = 4.7, p < 0.05$]. *Post hoc* analysis showed that: (1) the URB597-treated males had less intake than the vehicle-treated ones at 30% concentration [$p < 0.01$]; and (2) without URB597 treatment, mice had more intake at 30% concentration than at 7.5% [$p < 0.01$]. For preference ratio, two-way ANOVA revealed a significant effect of URB597 treatment [$F(1, 20) = 4.9, p < 0.05$], concentration [$F(1, 20) = 8.2, p < 0.01$], and a significant interaction between URB597 and concentration [$F(1, 20) = 5.5, p < 0.05$]. *Post hoc* analysis showed that the URB597-treated mice had less preference than the control ones at 30% concentration only [$p < 0.01$].

1.4. Pretreatment with selective CB1R antagonist AM251 (0.1 mg/kg) blocked the effect of acute URB597 (0.5 mg/kg) on reducing 15% alcohol drinking in male mice—One-way ANOVA revealed a significant effect of URB597 treatment [$F(3, 23) = 5.1, p < 0.01$], and *post hoc* analysis showed that the URB597-treated males had less intake than the vehicle-treated ones [$p < 0.05$]. Although pretreatment with AM251 at 0.1 mg/kg alone had no effect on alcohol drinking per se, it blunted the effect of URB597 at 0.5 mg/kg on alcohol drinking during 4 hours [$p < 0.05$] (Table 3). For preference ratio, one-way ANOVA revealed a significant effect of URB597 treatment [$F(3, 23) = 5.2, p < 0.01$], and *post hoc* analysis showed that (1) the URB597-treated males had less preference than the vehicle-treated ones [$p < 0.05$]; and (2) although pretreatment with AM251 at 0.1 mg/kg

alone had no effect on preference ratio per se, it blunted the reducing effect of URB597 [$p < 0.01$].

1.5. No effects of single, acute URB597 (0.5 mg/kg) on sucrose intake and preference in male mice—As alcohol is a caloric reinforcer, the specificity of the action of URB597 on alcohol intake was tested after single, acute administration of 0.5 mg/kg URB597 on sucrose (caloric reinforcer) drinking in mice after chronic IA. In these experiments, the chronic 15% alcohol exposure procedures were identical to those in the above experiments. After 3 weeks of chronic IA, the alcohol tube was switched to sucrose for 3 sessions over 6 days, with stable intake observed. The male mice assigned to the vehicle- or URB597-treated groups had similar sucrose intake 24 hours before the test day. URB597 had no significant effect on either intake or preference at 2% (Table 4A) or 4% (Table 4B) sucrose concentration. Further, in alcohol-naïve male mice ($n = 6$), there was no effect of 0.5 mg/kg URB597 on 4% sucrose intake (control mice: 7.5 ± 0.77 g/kg; URB597 mice: 7.7 ± 1.1 g/kg) or preference ratio (control: 0.93 ± 0.05 ; URB597: 0.96 ± 0.03).

1.6. Single, acute URB597 (0.5 mg/kg) increased saccharin preference ratio, but not intake in male mice—The specificity of the action of URB597 on alcohol intake was further tested on saccharin (non-caloric reinforcer) intake in mice. Like the sucrose experiments, after 3 weeks of chronic IA, the alcohol tube was switched to saccharin for 3 sessions over 6 days, and after stable intake observed, the male mice assigned to the vehicle-treated or URB597-treated groups had similar saccharin intake 24 hours before the test day. For saccharin intake after 4 hours (Figure 3A), two-way ANOVA revealed a significant effect of saccharin concentration [$F(1, 20) = 86$, $p < 0.00005$] only. *Post hoc* analysis showed that with or without URB597 treatment, mice had more intake at 0.2% concentration than at 0.1% [$p < 0.01$]. For preference ratio (Figure 3B), two-way ANOVA revealed a significant effect of URB597 treatment only [$F(1, 20) = 13$, $p < 0.005$]. *Post hoc* analysis showed that the URB597-treated mice had more preference than the control mice at both 0.1% and 0.2% concentrations [$p < 0.05$ for both]. Furthermore, in alcohol-naïve male mice ($n = 6$), there was no effect of 0.5 mg/kg URB597 on 0.1% saccharin intake (control mice: 0.15 ± 0.02 g/kg; URB597 mice: 0.13 ± 0.03 g/kg) or preference ratio (control: 0.95 ± 0.02 ; URB597: 0.97 ± 0.02).

2. Effect of single, acute or repeated administration of URB597 on ADE in both male and female mice

2.1. Single, acute URB597—In the single dose URB597 experiment, we tested the effect of URB597 at 0.5 mg/kg in both male and female mice, and found similar reducing effects on the ADE. As there were no significant sex differences in the effects of URB597, the data of each sex are presented separately (Figure 4).

In males at 4 hours (Figure 4A), two-way ANOVA revealed a significant effect of URB597 treatment [$F(1, 24) = 4.6$, $p < 0.05$], and a significant interaction between Session and Treatment [$F(1, 24) = 4.5$, $p < 0.05$], and *post hoc* analysis showed that the URB597-treated males had less intake than the vehicle-treated ones in the ADE session [$p < 0.05$]. To test our *a priori* hypothesis that there was an ADE, we included the *post-hoc* result showing that in

the vehicle-treated mice, the ADE was significant after 4 hours [$p < 0.05$], though 2-way ANOVA did not reveal any significant effect of Session after 4 hours (Figure 4A). There were no significant effects of ADE or acute URB597 after 8 or 24 hours (data not shown).

In females at 4 hours (Figure 4B), two-way ANOVA revealed a significant effect of Session [$F(1, 26) = 4.3$, $p < 0.05$], and *post hoc* analysis showed that the females had more intake in the ADE session than the baseline [$p < 0.05$]. Though overall Treatment difference was not significant, *post hoc* analysis showed that the URB597-treated females had less intake than the vehicle-treated ones in the ADE session [$p < 0.05$] (Figure 4B). Similar to the male mice, there were no significant effects of ADE or acute URB597 treatment after 8 or 24 hours in females (data not shown).

2.2. Repeated URB597—In the repeated URB597 experiment, we tested the effect of URB597 at 0.5 mg/kg in both male and female mice, and found similar reducing effects on the ADE. As there were no significant sex differences in the effects of URB597, the data of each sex are presented separately (Figure 5).

In males at 4 hours (Figure 5A), two-way ANOVA revealed a significant effect of URB597 treatment [$F(1, 20) = 5.8$, $p < 0.05$], and a significant interaction between Session and Treatment [$F(1, 20) = 5.9$, $p < 0.05$], and *post hoc* analysis showed that the URB597-treated males had less intake than the vehicle-treated ones in the ADE session [$p < 0.05$]. To test our *a priori* hypothesis that there was an ADE, we included the *post-hoc* result showing that in the vehicle-treated mice, the ADE was significant after 4 hours [$p < 0.05$] (Figure 5A), though 2-way ANOVA did not reveal any significant effect of Session. There were no significant effects of ADE or repeated URB597 after 8 or 24 hours (data not shown).

In females at 4 hours (Figure 5B), two-way ANOVA revealed a significant effect of Session [$F(1, 26) = 15.1$, $p < 0.001$] and *post hoc* analysis showed that the females had more intake in the ADE session than the baseline [$p < 0.01$]. However, there was no significant effect of the repeated URB597 treatment after 8 or 24 hours (data not shown).

3. Acute (1 day) alcohol withdrawal transiently caused global brain eCB system dysregulation in male mice

3.1. Acute (1 day) withdrawal—Relative NAE abundances were detected in four mouse brain regions from male mice subjected to the chronic IA model following 1 day alcohol withdrawal as well as from control (water) male mice. All four brain regions showed a significant increase in the NAEs (Figure 6, S1). AEA levels were increased the greatest, with a 3.1, 2.7, 2.1 and 1.9 -fold increase in the NAc, BLA, cerebellum and PFC, respectively (Figure 6A). Palmitoyl ethanolamide (PEA) and oleoyl ethanolamide (OEA) were similarly increased (Figure 6B, 6C, S1), as were other NAEs (data not shown).

3.2. Chronic IA and long-term withdrawal—Relative NAE abundances were further detected in the BLA of mice subjected to the chronic IA model as well as from that of control (water) mice in three groups: immediately after 3 week IA and long-term (1- and 2-week) withdrawal. This was done in order to determine: (A) whether the increased NAEs after 1 day withdrawal were due to chronic alcohol drinking or due to acute withdrawal, and

(B) if NAE elevation after acute withdrawal was a short-term response to withdrawal or whether it persisted. Neither of these groups showed changed NAE levels, indicating that NAE elevation was a transient response to acute withdrawal (Figure 7A, S2). As presented as fold change of AEA (Figure 7B), one-way ANOVA revealed a significant effect of time [$F(3, 20) = 109, p < 0.000001$], and *post hoc* analysis showed that the 1-day (24 hours) withdrawal had higher ADE levels than other time points examined [$p < 0.0001$].

4. FAAH levels in the prefrontal cortex after withdrawal from chronic IA in male mice

Protein lysates prepared from the PFC of male mice were analyzed by Western Blotting. No difference in FAAH levels was detected after acute (1 day) or long-term (1 and 2 weeks) withdrawal, as compared to the water control (Figure 8).

DISCUSSION

Our first objective in the present study was to investigate the potential of an FAAH inhibitor, URB597, in reducing alcohol consumption in mice after acute (1 day) withdrawal from chronic IA alcohol drinking. Single acute administration of URB597 (a long-lasting inhibitor) significantly reduced 15% alcohol intake in mice in a dose-dependent manner (0.125–1 mg/kg) (Figure 2A). The URB597-induced reduction of alcohol intake was coupled with a full compensatory increase in water intake, resulting in no change in total fluid intake (Table S1). This indicates the absence of nonspecific sedative effects by this FAAH inhibitor. Consistently, URB597 (0.125–1 mg/kg) produced a dose-dependent reduction in alcohol preference (Figure 2B). In the present study, we further confirmed that URB597 reduced alcohol drinking in a CB1-dependent manner, as selective CB1 antagonist AM251 blocked the effects of URB597 (Table 3). It is unlikely that the effect of URB597 in reducing alcohol intake was secondary to a general suppression of appetitive (anhedonic effect) or consumption behaviors, since URB597 at 0.5 mg/kg did not decrease sucrose (caloric reinforcer) (Table 4) or saccharin (non-caloric reinforcer) (Figure 3) intake. The present study also found a relatively long duration (4 hours) of the effect of single acute URB597 administration on alcohol drinking, which is likely due to its long-lasting bioactivity *in vivo* [Fegley et al. 2005; Basavarajappa et al. 2014]. Together, these results clearly demonstrate that the URB597-induced inhibition of FAAH plays a role in modulating alcohol drinking in mice. Therefore, the development of FAAH inhibitors with improved pharmacokinetics has the potential to yield useful compounds for the treatment of alcoholism and other drug addictions [Panlilio et al. 2013; Zhou & Kreek 2014].

Though URB597 does not produce any reward [Gobbi et al. 2005], repeated administration of URB597 has been found to correct the reduction in sucrose intake after chronic stress in both mice [Rademacher & Hillard, 2007] and rats [Bortolato et al. 2007]. Our results showed that single acute URB597 administration at 0.5 mg/kg (the most effective dose tested for reducing alcohol intake) had no effect on sucrose or saccharin preference in alcohol naïve mice, but increased saccharin preference in mice after 1-week alcohol withdrawal (Figure 3). Consistently, previous work has found that URB597 does *not* affect saccharin or sucrose drinking in stress-naïve animals (not rewarding) [Basavarajappa et al. 2006; Blednov et al. 2007; Manwell et al. 2009], but increases sucrose preference and/or

intake in stress exposed animals due to “anti-depression” properties [Rademacher & Hillard, 2007; Bortolato et al. 2007]. Together, our data suggest that the enhanced eCB activity resulting from the FAAH inhibitor can modulate sensitivity to saccharin reward during alcohol withdrawal. Alternatively, URB597 as an indirect eCB agonist may increase preference for more palatable reinforcers, such as saccharin (Figure 3) and sucrose (slight, but not significant, increases at 2% [Table 4A]), with decreased preferences for less palatable reinforcers (15% and 30 % alcohol), though URB597 itself is not rewarding [Gobbi et al. 2005].

With low alcohol intake models, previous studies tested the FAAH inhibitor URB597 on alcohol-related behaviors: [1] on reinstatements of alcohol-seeking behavior, no effect was found after acute URB597 administration in rats with alcohol intake < 2 g/kg during 1 hour of alcohol access [Cippitelli et al. 2008]; and [2] URB597 was reported to increase alcohol drinking in mice with alcohol intake < 4 g/kg/day during 24 hours of alcohol access [Blednov et al. 2007]. In contrast to mice in the 4-hour limited-access DID paradigm, which had low alcohol intake (<5–6 g/kg/day) [Rhodes et al. 2007], mice in the chronic IA paradigm showed “heavy” drinking with 15–20 g/kg/day in males and 20–25 g/kg/day in females [Hwa et al. 2011; Zhou et al. 2017]. For these reasons, we purposely compared the effects of URB597 on chronic IA with DID, and found that single acute URB597 administration at 0.5 mg/kg reduced alcohol drinking after 1 day withdrawal from chronic IA (Figure 2), with no effect after 1 day withdrawal from chronic DID (SI section, Results S1). When tested in other alcohol concentrations, we observed that single acute administration of URB597 at 0.5 mg/kg reduced high levels of alcohol intake with 30% alcohol, but had no effect on low levels of alcohol intake with 7.5% alcohol (Table 2), consistent with the above notion. Together, our results demonstrate that URB597 modulates alcohol drinking in mice with long-access and chronic exposure to high levels of alcohol intake, but not with either limited access (like DID) or low levels of alcohol intake at 7.5% concentration. In accord with previous research, our data further indicate that the protective role of the eCBs becomes effective as the withdrawal from “heavy” alcohol drinking is pronounced [Morena et al. 2016]. Drug escalation to high level intake is widely considered a hallmark of the transition from alcohol abuse to drug dependence in humans. Thus, our preclinical study may provide new information about the medical potential of URB597 in the treatment of alcoholism.

Our second main objective was to investigate the potential of URB597 in reducing relapse-like drinking in mice after long-term withdrawal. The phenomenon of a transient increase in alcohol consumption after a period of imposed abstinence has been termed the alcohol deprivation effect (ADE). It can be regarded as an animal model of relapse behavior and craving with good predictive validity [Vengeliene et al. 2014]. In the present study, mice of both sexes displayed the ADE with increased alcohol intake after 1 week of withdrawal (Figures 4, 5). In contrast, the mice pretreated with single acute URB597 administration at 0.5 mg/kg had a blunted ADE in both sexes (Figures 4), suggesting the involvement of FAAH activity in the relapse-like drinking behavior. This finding with alcohol would constitute an interesting extension to the anti-relapse properties of URB597 observed in cocaine [Chauvet et al. 2014], nicotine [Scherma et al. 2008] or opiate seeking behaviors [Manwell et al. 2009]. One concern about the use of URB597 is that its FAAH inhibitory

activity after repeated administration could result in the development of tolerance. Therefore, we tested the effectiveness of the multiple-dosing regimen (5 daily injections before the ADE during the 1-week withdrawal) to mimic the multiple-dosing treatment in the clinic. Like the effect of single acute URB597 administration at 0.5 mg/kg on reducing the ADE in both sexes (Figures 4), the repeated pretreatments of URB597 at the same dose during the withdrawal week efficiently prevented the ADE drinking in mice (Figure 5), showing no tolerance development after this multiple-dosing regimen. It is worth mentioning that the prevention of the ADE by this multiple-dosing regimen occurred with the last URB597 administration 1 day before alcohol re-exposure ADE. Thus, our findings show promising *in vivo* data indicating that the long-lasting FAAH inhibitor URB597 may show therapeutic potential in alcoholism treatment models.

Previous lines of experimental evidence have suggested chronic alcohol-exposed rats and mice as an animal model of alcohol withdrawal-induced “anxiety” and “depression”. These emotional states, and the search for the anxiolytic and antidepressant-like effects of alcohol, might in turn contribute to promoting the high levels of alcohol intake and preference that characterize the escalation drinking observed in the present study in mice [Colombo et al. 1995; Koob and Volkow 2010]. The stress-response eCB system is critically involved in “anxiety”-related behaviors [Dincheva et al. 2015; Parsons & Hurd 2015; Pardini et al. 2012; Spagnolo et al. 2016], and FAAH inhibition reverses enhanced anxiety-like behavior present during alcohol withdrawal [Cippitelli et al. 2008]. Our mouse escalation model suggests a role for FAAH activity in the alcohol withdrawal-induced tendency of mice towards “anxiety”- and “depression”- like states. Thus, the increased AEA/CB1 activity after URB597 administration, by inhibiting FAAH activity [Basavarajappa et al. 2014], could be partially responsible for the anxiolytic action of the FAAH inhibitor and subsequent reduction in alcohol intake after acute withdrawal in both male and female mice (Figure 1). In support of this concept, CB1 knockout mice with higher “anxiety”-like behavior [Bowers & Ressler 2016] display higher alcohol consumption than the wild-type littermates during the first days [Racz et al. 2003].

Therefore, we sought to determine the effects of chronic high alcohol intake and subsequent alcohol withdrawal on the eCB system at a molecular level to better understand the dynamic changes in different brain regions. We used a targeted mass spectrometry method to quantify the relative abundance of NAE family of eCBs in the NAc, BLA, PFC and cerebellum of mice. Interestingly, after acute 1-day withdrawal from 3-week chronic IA alcohol drinking, all four brain regions showed significant increases in the AEA, OEA and PEA, with the NAc and BLA displaying the largest changes (Figure 6). As previous studies showed alterations of AEA levels in the amygdala and other brain regions of rats after chronic alcohol or withdrawal [Rubio et al. 2008; Robinson et al. 2016; Henricks et al. 2017], we further determined dynamic profiles of relative abundances of NAEs in the BLA following chronic IA drinking (no withdrawal) and after one and two weeks of alcohol withdrawal, all of which showed no change compared to control (Figure 7A, S2). Together, this indicates that global NAE dysregulation occurs as a response to acute alcohol withdrawal only. This transient increase may be due to decreased FAAH activity, as found in the rats after 1-day alcohol withdrawal [Serrano et al. 2012], but not the FAAH expression level as observed in the present study (Figure 8). To our knowledge, this is the first description of global NAE

dysregulation, both in terms of multi-localization and number of endogenous substrates (AEA, OEA, and PEA), in response to acute alcohol withdrawal, and indicates that a strong eCB response may act as an important molecular coping mechanism in acute alcohol withdrawal, thereby allowing the mice to better cope with alcohol withdrawal stress. As a possible allostatic process, the AEA surges may be resultant from the absence of an AEA release during 1-day acute withdrawal. Indeed, a recent study has found that the AEA release in the rat nucleus accumbens is observed only after acute alcohol, but not chronic alcohol or chronic withdrawal [Ceccarini et al. 2013], though other reports showed that acute alcohol decreases [Ferrer et al. 2007] or did not change AEA release in this region [Alvarez-Jaimes et al. 2009]. AEA and other NAE elevations were detected at 1-day withdrawal, whereas behavioral responses to the FAAH inhibitor URB597 (inhibition of alcohol drinking) were apparent at both 1-day and 1-week withdrawal time points. With the available data, the best explanation of this phenomenon is that naturally or pharmacologically elevated NAEs are protective from alcohol withdrawal stress. Naturally, this response (observed in tissue content, but not release) occurs only transiently as an acute response to withdrawal and does not persist after a week. However, after one week of alcohol withdrawal, pharmacological elevation of NAEs with an FAAH inhibitor still provides the same anti-stress protection, leading to reduce “relapse” drinking. As the role of eCBs is complicated by their involvement in modulation of alcohol escalation and “relapse” drinking, two other possible explanations should be considered. The AEA changes in other brain regions rather than the four areas tested could be correlated to alcohol withdrawal. Alternatively, our data suggest that the CB1 binding and/or function, and the downstream effects of CB1 receptor signaling can be more involved in the behavioral effects of the FAAH inhibition. Further study to elucidate these findings is warranted. In summary: In line with previous studies on cocaine, nicotine and opiate seeking behaviour [Panlilio et al. 2013], our findings show initial, promising *in vivo* data indicating that pharmacological FAAH inhibition decreases alcohol escalation and “relapse” drinking in mice.

Supplementary Material

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Acknowledgments

This work was supported by NIH AA021970 (YZ), NS095698 (BIS), NHLBI 528915035 (SSG), Pritzker Neuropsychiatric Disorders Research Consortium (FSL) and Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (MJK).

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A and B, Male**A: 15% Alcohol Intake** **B: 15% Alcohol Preference Ratio** **C and D, Female****C: 15% Alcohol Intake** **D: 15% Alcohol Preference Ratio** **Figure 1.**

Effects of single, acute administration of URB597 (URB, 0.5 mg/kg) on 15% alcohol intake and preference ratio in male (A and B, n=7–8) and female (C and D, n=8) mice after 1 day of withdrawal from 3-week chronic intermittent access alcohol drinking. Alcohol and water intake values were recorded after 4, 8 and 24 hours of alcohol access. * $p < 0.05$ and ** $p < 0.01$ vs. vehicle control at the same time point.

Figure 2.

Dose responses of single, acute administration of URB597 (URB, 0, 0.125–1 mg/kg) on reducing 15% alcohol intake (A) and preference ratio (B) in male mice (n=6–7) after 1 day of withdrawal from 3-week chronic intermittent access alcohol drinking. Data were collected at the 4-hour time point on the testing day and are expressed as a percentage of alcohol intake in vehicle-treated mice. * $p < 0.05$ or ** $p < 0.01$ vs. control (URB597 at 0 mg/kg), and + $p < 0.05$ vs. 0.125 or 0.25 dose.

Figure 3.

Effects of single, acute administration of URB597 (URB, 0.5 mg/kg) on 0.1% or 0.2% saccharin intake (**A**) and preference ratio (**B**) in male mice (n=6) after 3-week chronic intermittent access alcohol drinking. Saccharin and water intake values were recorded after 4 hours of saccharin access. * $p < 0.05$ vs. vehicle control at the same saccharin concentration, and ++ $p < 0.01$ vs. 0.1% saccharin.

A. Male **B. Female** **Figure 4.**

Effects of single, acute administration of URB597 (URB, 0.5 mg/kg) on alcohol intake in an alcohol deprivation effect (ADE) model at 4 hours in male (A, n=7) and female (B, n=6–9) mice after 1 week of withdrawal from 3-week chronic intermittent access alcohol drinking. * $p < 0.05$ vs. control baseline, and + $p < 0.05$ vs. ADE.

Figure 5.

Effects of five repeated administration of URB597 (URB, 0.5 mg/kg) on alcohol intake in an alcohol deprivation effect (ADE) model at 4 hours in male (A, n=6) and female (B, n=7–8) mice after 1 week of withdrawal from 3-week chronic intermittent access drinking. During the withdrawal, mice received five daily URB597 or vehicle injections with the last one 1 day before alcohol bottles were presented again. * $p < 0.05$ vs. control baseline, and + $p < 0.05$ vs. ADE.

Figure 6.

Effects of 1-day withdrawal from 3-week chronic intermittent access (IA) alcohol drinking on brain NAE abundances in male mice (n=6). Test mice are labelled Alcohol and control mice are labelled Water. (A) Anandamide (AEA) abundances in four brain regions after 1 day alcohol withdrawal. (B) Palmitoyl ethanolamide (PEA) and oleoyl ethanolamide (OEA) abundances in the basolateral amygdala after 1 day alcohol withdrawal. Normalized, relative abundances are shown in all graphs. * $p < 0.05$ ** $p < 0.001$ and *** $p < 0.0001$ vs. control, error bars indicate standard error of the mean.

Figure 7.

Anandamide (AEA) abundances after chronic 3-week chronic intermittent access (IA) alcohol drinking or long-term withdrawal in male mice. (A) AEA abundances after chronic IA (labelled "none"), 1-week or 2-week withdrawal; Alcohol naïve control mice labelled Water; (B) AEA abundances presented as fold change vs. control for each tested time point. Normalized, relative abundances are shown in all graphs. *** $p < 0.0001$ vs. other time points, error bars indicate standard error of the mean.

Figure 8.

No FAAH changes in male mice (n=6) after acute or long-term withdrawal from 3-week chronic intermittent access (IA) alcohol drinking. A Western blot of FAAH at each time point and the corresponding intensity are shown. Control mice were given only water. Test mice were exposed to chronic IA alcohol (15%) drinking for 3 weeks and then 1 day, 1 week or 2 week of withdrawal.

Table 1

Experimental timelines

A. The 3-week chronic intermittent access (IA) alcohol drinking model (15% alcohol vs. water, 24 hours) every other day with selective FAAH inhibitor URB597, CB1 antagonist AM251 or their combination.			
Week 1	Week 2	Week 3	1-day withdrawal (day 22)
IA 24-h alcohol vs water	IA 24-h alcohol vs water	IA 24-h alcohol vs water	Single URB597 with AM251 24-h alcohol vs water

B. The 3-week chronic alcohol drinking-in-the-dark (DID) model (15% alcohol, 4 hours) every day with selective FAAH inhibitor URB597.			
Week 1	Week 2	Week 3	1-day withdrawal (day 22)
4-h alcohol	4-h alcohol	4-h alcohol	Single URB597 4-h alcohol

C. The alcohol deprivation effect (ADE) model after 1-week withdrawal from a 3-week chronic intermittent access (IA) drinking (15% alcohol vs. water) with selective FAAH inhibitor URB597.				
Week 1	Week 2	Week 3	Week 4	Day 30
IA 24-h alcohol vs water	IA 24-h alcohol vs water	IA 24-h alcohol vs water Baseline (BL)	1-week withdrawal 5 repeated URB597	Single URB597 ADE

Table 2

Effects of single, acute administration of URB597 (URB, 0.5 mg/kg) on 7.5% or 30% alcohol intake and preference ratio at 4 hours in male mice after 1 day of withdrawal from 3-week chronic intermittent access (IA) alcohol drinking.

	7.5% alcohol		30% alcohol	
	Vehicle (n = 6)	0.5 mg/kg URB (n = 6)	Vehicle (n = 6)	0.5 mg/kg URB (n = 6)
Intake, g/kg	3.6 ± 0.45	2.7 ± 0.40	11 ± 0.24 ⁺⁺	4.7 ± 0.81 ^{**}
Preference ratio	0.75 ± 0.05	0.76 ± 0.08	0.70 ± 0.08	0.40 ± 0.06 ^{**}

*
p<0.05 or

**
p<0.01 vs. vehicle control at the same alcohol concentration, and

⁺⁺
p<0.01 vs. vehicle group at 7.5% alcohol.

Table 3

Pretreatment with selective CB1R antagonist AM251 (0.1 mg/kg) blocks the effect of single, acute URB597 (URB, 0.5 mg/kg) on reducing 15% alcohol drinking in male mice after 4 hours of alcohol access.

	Vehicle + vehicle (n = 8)	Vehicle + URB (n = 6)	AM251 + vehicle (n = 7)	AM251 + URB (n = 6)
Intake, g/kg	5.6 ± 0.65	3.6 ± 0.41 *	5.1 ± 0.25	4.8 ± 0.41 ⁺
Preference	0.81 ± 0.07	0.55 ± 0.04 *	0.82 ± 0.05	0.74 ± 0.04 ⁺⁺

* p<0.05 vs. vehicle + vehicle control;

⁺ p<0.05 or

⁺⁺ p<0.01 vs. vehicle + URB.

Table 4

No effect of single, acute administration of URB 597 (URB, 0.5mg/kg) on 2% (A) and 4% (B) sucrose intake and their preference ratio in male mice after 4 hours of sucrose access.

A		
2% sucrose	Vehicle (n = 6)	0.5 mg/kg URB (n = 6)
Intake, g/kg	1.69 ± 0.14	2.10 ± 0.26
Preference	0.89 ± 0.04	0.94 ± 0.02

B		
4% sucrose	Vehicle (n = 6)	0.5 mg/kg URB (n = 6)
Intake, g/kg	7.10 ± 0.98	7.70 ± 0.70
Preference	0.94 ± 0.02	0.93 ± 0.02