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Authors

Cippitelli, Andrea
Cannella, Nazzareno
Braconi, Simone
et al.

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ORIGINAL INVESTIGATION

Increase of brain endocannabinoid anandamide levels by FAAH inhibition and alcohol abuse behaviours in the rat

Andrea Cippitelli · Nazzareno Cannella · Simone Braconi · Andrea Duranti · Andrea Tontini · Ainhoa Bilbao · Fernando Rodríguez DeFonseca · Daniele Piomelli · Roberto Ciccocioppo

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Abstract
Rationale A major clinical concern with the use of cannabinoid receptor 1 (CB1) direct agonists is that these compounds increase alcohol drinking and drug abuse-related behaviours. As an alternative approach, CB1-receptor-mediated activity can be facilitated by increasing anandamide levels with the use of hydrolase fatty acid amide hydrolase (FAAH) inhibitors.
Objective Using the selective FAAH inhibitor URB597, we investigated whether activation of the endogenous cannabinoid tone increases alcohol abuse liability, as what happens with the CB1 receptor direct agonists.
Materials and methods URB597 was tested on alcohol self-administration in Wistar rats and on homecage alcohol drinking in genetically selected Marchigian Sardinian alcohol-preferring (msP) rats. In Wistar rats, URB597 effects on alcohol-induced anxiety and on stress-, yohimbine- and cue-induced reinstatement of alcohol seeking were also evaluated. For comparison, the effect of the CB1 receptor antagonist rimonabant on ethanol self-administration was also tested.
Results Under our experimental condition, intraperitoneal (IP) administration of URB597 (0.0, 0.3 and 1.0 mg/kg) neither increased voluntary homecage alcohol drinking in msP rats nor facilitated fixed ratio 1 and progressive ratio alcohol self-administration in nonselected Wistars. In the reinstatement tests, the compound did not have effects on cue-, footshock stress- and yohimbine-induced relapse. Conversely, URB597 completely abolished the anxiogenic response measured during withdrawal after an acute IP administration of alcohol (3.0 g/kg). Rimonabant (0.0, 0.3, 1.0 and 3.0 mg/kg) significantly reduced ethanol self-administration.
Conclusions Results demonstrate that activation of the endocannabinoid anandamide system by selective inhibition of FAAH does not increase alcohol abuse risks but does reduce anxiety associated to alcohol withdrawal. We thus can speculate that medication based on the use of endocannabinoid system modulators such as URB597 may offer important advantages compared to treatment with direct CB1 receptor activators.

Keywords Anandamide · Cannabinoids · URB597 · FAAH · Alcohol drinking · Relapse · Alcohol self-administration

A. Cippitelli · N. Cannella · S. Braconi · R. Ciccocioppo (✉)
Department of Experimental Medicine and Public Health,
University of Camerino,
Via Madonna delle Carceri,
62032 Camerino, Italy
e-mail: roberto.ciccocioppo@unicam.it

A. Duranti · A. Tontini
Institute of Medicinal Chemistry,
University of Urbino "Carlo Bo",
Piazza del Rinascimento 6,
61029 Urbino, Italy

A. Bilbao · F. R. DeFonseca
Fundación IMABIS, Hospital Carlos Haya de Málaga,
Avenida Carlos Haya 82,
29010 Malaga, Spain

D. Piomelli
Department of Pharmacology,
360 MSRII, University of California,
Irvine, CA 92697-4625, USA

Introduction

The endocannabinoid system is considered one of the major pharmacological targets for the development of new therapeutics. For example, there are indications supporting the use of cannabinoid receptor 1 (CB1) agonists in the treatment of neuropatic pain, multiple sclerosis, anxiety, glaucoma, nausea and vomiting (Pacher et al. 2006). One of the major concerns with the use of CB1 receptor agonists comes from results showing that these compounds may have abuse potential and increase intake of other drugs of abuse, especially alcohol. It is known, for example, that in rodents, direct activation of CB1 receptors by CP 55,940 and WIN 55,212-2 dose dependently increase voluntary alcohol consumption (Colombo et al. 2002, 2004) and alcohol drinking motivation (Gallate et al. 1999), while Δ^9-THC significantly reinstates responding previously reinforced with beer (McGregor et al. 2005). Administration of the CB1 receptor agonist WIN 55,212-2 during abstinence was also shown to increase alcohol consumption when the alcoholic solution was made available again (López-Moreno et al. 2004).

Anandamide is the best characterised endogenous ligand for cannabinoid CB1 receptors (Devane et al. 1992; Piomelli 2003); it is released on demand by membrane lipids of stimulated post-synaptic neurons (Giuffrida et al. 1999), and, acting through a retrograde mechanism, it activates CB1 receptors onto pre-synaptic neurons to suppress subsequent neurotransmitter release (Wilson and Nicoll 2002; Freund et al. 2003). Anandamide is eliminated through a two-step process consisting of uptake into cells mediated by a transporter (Beltramo et al. 1997) and hydrolysis catalysed by the membrane-bound serine hydrolase fatty acid amide hydrolase (FAAH; Cravatt et al. 1996). Blockade of FAAH increases endocannabinoid transmission with a mechanism that does not require direct activation of cannabinoid receptors via synthetic cannabinoid agonists.

URB597 is a potent, selective and systemically active inhibitor of FAAH (Kathuria et al. 2003; Fegley et al. 2005) that increases endogenous anandamide levels and facilitates endocannabinoid neurotransmission (Kathuria et al. 2003). Recently, this compound was shown to exert profound anxiolytic- and antidepressant-like activities in rodents; these activities were prevented by the CB1 receptor antagonist rimonabant and were not accompanied by overt rewarding effects (Kathuria et al. 2003; Gobbi et al. 2005). These data suggest that activation of the endocannabinoid system through inhibition of anandamide degradation can offer an alternative strategy to stimulate cannabinoid neurotransmission while avoiding the psychotropic effects of CB1 receptor direct agonists (Piomelli 2003). However, at present, it is unclear whether increased anandamide levels after FAAH inhibition may stimulate alcohol drinking or facilitate reinstatement of alcohol seeking during abstinence, as do CB1 receptor direct agonists. In order to evaluate the safety profile of FAAH inhibitors and to understand if pharmacotherapeutic approaches based on the use of indirect CB1 receptor activators may offer advantages over direct receptor stimulation, we investigated the effect of URB597 on alcohol consumption and on reinstatement of alcohol-seeking behaviour following presentation of cues predictive of drug availability or following exposure to stress.

Materials and methods

Animals

Male Wistar rats weighing 175–225 g at the beginning of the experiments and genetically selected alcohol-preferring Marchigian Sardinian (msP) rats weighting 400–450 g were used. Wistar rats were purchased from Charles River, while msP rats were bred at the Department of Pharmacological Sciences and Experimental Medicine of the University of Camerino (Italy) for more than 58 generations from Sardinian alcohol-preferring rats of 13th generation provided by the Department of Neurosciences of the University of Cagliari (Colombo et al. 2006) in a temperature- and humidity-controlled vivarium and on a reverse 12-h light / dark cycle (lights on 20:00–08:00 h). MsP rats were individually housed, whereas Wistars were kept in pairs. All training and experimental sessions were conducted during the nocturnal phase of the light/dark cycle. Standard laboratory rat chow (Mucedola, Settimo Milanese) and water were available ad libitum in the homecage. Each experiment was conducted with independent groups of rats. All procedures were conducted in adherence to the European Community Council Directive and the National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

Drugs

URB597 was synthesised by the Institute of Medicinal Chemistry of the University of Urbino "Carlo Bo" as previously described by Mor et al. (2004). It was suspended in 5% PEG400, 5% TWEEN80 and 90% saline. Rimonabant (SR-141716A) was obtained from Sanofi-Synthelabo Recherche (Montpellier, France). The compounds were administered intraperitoneally (IP) at a volume of 1 ml/kg. The doses used were based on previous studies (Kathuria et al. 2003; Gobbi et al. 2005). Yohimbine HCl, purchased from Sigma (Italy), was dissolved in distilled water in a dose chosen on the basis of previous studies (Shepard et al. 2004; Lê et al. 2005) and injected IP (1 ml/kg).

Operant training

Training and testing were conducted in standard operant chambers (Med Associate) located in sound-attenuating, ventilated environmental cubicles. Each chamber was equipped with a drinking reservoir (volume capacity: 0.30 ml) positioned 4 cm above the grid floor in the centre of the front panel of the chamber and two retractable levers located 3 cm to the right or to the left of the drinking receptacle. Auditory and visual stimuli were presented via a speaker and a light located on the front panel. A microcomputer controlled the delivery of fluids, presentation of auditory and visual stimuli and recording of the behavioural data. Rats were trained to self-administer 10% alcohol (*v*/*v*) in 30-min daily sessions on a fixed ratio 1 schedule of reinforcement, in which each response resulted in delivery of 0.1 ml of fluid as previously described (Economidou et al. 2006). For the first 3 days, rats were allowed to lever-press for a 0.2% (*w*/*v*) saccharin solution, and then trained to self-administer 10% alcohol by fading the saccharine (Weiss et al. 1993). During the first 6 days of training, rats were allowed to lever-press for a 5.0% (*v*/*v*) alcohol solution containing 0.2% (*w*/*v*) saccharin. Starting on day 7, the concentration of alcohol was gradually increased from 5.0% to 8.0% and finally to 10.0% (*w*/*v*), while the concentration of saccharin was correspondingly decreased to 0%.

Alcohol self-administration

Following completion of the saccharin fading procedure, Wistar rats (n=18) were trained to 30-min 10% alcohol (0.1 ml per response) self-administration sessions under a fixed ratio 1 (FR1) schedule of reinforcement. Alcohol self-administration sessions were performed until a stable baseline of responses was reached. One group of rats (n=8) was treated with URB597 (0.0, 0.1, 0.3 and 1.0 mg/kg), the second group received rimonabant (0.0, 0.3, 1.0 and 3.0). Since FAAH inhibition occurs rapidly (<15 min) after administration of URB597, we gave the drug 30 min prior to the self-administration session (Kathuria et al. 2003). Based on existing data, rimonabant was also given 30 min before the beginning of the session (Economidou et al. 2006). Experiments were conducted every fourth day using a Latin square counterbalanced design. Responses at the inactive lever were recorded throughout the experiment to monitor non-specific behavioural effects.

Progressive ratio schedule of reinforcement

In this experiment, Wistar rats (n=14) were tested under a progressive ratio schedule of reinforcement to measure the break point (the last ratio completed by the animals) for alcohol (Arnold and Roberts 1997). For this purpose, animals were first trained to self-administer 10% alcohol under a FR1 schedule of reinforcement. Following acquisition of a stable baseline of responding for alcohol, rats were tested under the progressive ratio (PR) conditions in which the response requirement (i.e. the number of lever responses or the ratio required to receive one dose of 10% alcohol) was increased as follows. For each of the first four alcohol deliveries, the ratio was increased by 1; for the next four deliveries, the ratio was increased by 2; for all the following deliveries, the ratio was increased by 4. Each alcohol-reinforced response resulted in a 1.0-s illumination of the house light, while sessions were terminated when more than 30 min had elapsed since the last reinforced response (Ciccocioppo et al. 2004). URB597 1.0 mg/kg or vehicle was given 30 min before starting the operant conditioning experiment.

Voluntary alcohol intake

At the beginning of the experiment, msP rats (n=9) were allowed free choice between water and 10% (*v*/*v*) alcohol 24 h/day for 5 days. Starting on day 6 and for the following 15 days, access to alcohol was limited to a period of 60 min/day at the beginning of the dark phase. Water and alcohol were offered in graduated drinking tubes equipped with metallic drinking spouts. The position (to the right or left) of alcohol and water drinking tubes was changed daily to avoid the development of side preference. Water and food were available ad libitum, while alcohol access was restricted to 1 h. The effect of URB597 (0.0, 0.1, 0.3, 1.0 mg/kg) given 30 min prior to the exposure to alcohol was studied. Experiments were conducted every fourth day using a Latin square counterbalanced design. Alcohol, water and food intakes were measured during the 1 h of limited alcohol access.

Cue-induced reinstatement of alcohol-seeking behaviour

Conditioning phase

At completion of the fading procedure (see above), in 30-min daily sessions, animals were trained to discriminate between 10% alcohol and water. Beginning with self-administration training at the 10% alcohol concentration, discriminative stimuli (SD) predictive of alcohol versus water availability were presented during the alcohol and water self-administration sessions, respectively. The discriminative stimulus for alcohol consisted of the odour of an orange extract (S^+), whereas water availability (i.e. no reward) was signalled by an anise extract (S^-). The olfactory stimuli were generated by depositing six to eight drops of the respective extract into the bedding of the operant chamber. In addition, each lever-press

resulting in delivery of alcohol was paired with illumination of the chamber's house light for 5 s (CS^+). The corresponding cue during water sessions was a 5-s tone (70 dB; CS^-). Concurrently with the presentation of these stimuli, a 5-s time-out period was in effect, during which responses were recorded but not reinforced. The olfactory stimuli serving as S^+ or S^- for alcohol availability were introduced 1 min before extension of the levers and remained present throughout the 30-min sessions. The bedding of the chamber was changed, and bedding trays were cleaned between sessions. During the first 3 days of the conditioning phase, the rats were given alcohol sessions only. Subsequently, alcohol and water sessions were conducted in random order across training days, with the constraint that all rats received a total of ten alcohol and ten water sessions.

Extinction phase

After the last conditioning day, rats were subjected to 30-min extinction sessions for 15 consecutive days. During this phase, sessions began by extension of the levers without presentation of the SD. Responses at the lever activated the delivery mechanism but did not result in the delivery of liquids or the presentation of the response-contingent cues (house light or tone).

Reinstatement testing

Reinstatement tests began the day after the last extinction session. This test lasted 30 min under conditions identical to those during the conditioning phase, except that alcohol and water were not made available. Sessions were initiated by the extension of both levers and presentation of either the alcohol S^+ or water S^- paired stimuli. The respective SD remained present during the entire session, and responses at the previously active lever were followed by activation of delivery mechanism and a 5-s presentation of CS^+ in the S^+ condition or the CS^- (tone) in the S^- condition. Wistar rats (n=9) were tested under the S^+/CS^+ condition on day 1 and under the S^-/CS^- condition on day 2. Subsequently, reinstatement experiments were conducted every fourth day (on days 6, 10, 14, 18). In a counterbalanced order, URB597 (0.0, 0.1, 0.3 and 1.0 mg/kg) was administered IP 30 min prior to the sessions. Responding at the inactive lever was also recorded to monitor possible non-specific behavioural effects.

Footshock stress- and yohimbine-induced reinstatement of alcohol seeking

In reinstatement studies, electric footshock is commonly used to facilitate resumption of drug use following extinction (Lê et al. 1998; Lê and Shaham 2002). Reinstatement of drug seeking is also obtained with administration of the α-2 adrenoreceptor antagonist yohimbine, which, by increasing brain noradrenaline cell firing (Aghajanian and VanderMaelen 1982) and release (Abercrombie et al. 1988), acts as a pharmacological stressor (Bremner et al. 1996a,b; Charney et al. 1983; Holmberg et al. 1962; Lee et al. 2004; Lê et al. 2000, 2005). These two methods were used to investigate the effect of URB597 on stress-induced alcohol seeking.

Training phase

At completion of the fading procedure, Wistar rats (n=48) were trained to self-administer 10% (v/v) alcohol for 15 days in 30-min daily sessions under a FR1 schedule of reinforcement. During the infusion, a stimulus house light was turned on for 5 s (time out (TO)). Lever presses during the TO period were counted but did not lead to further infusions.

Extinction phase

After the last alcohol self-administration session, animals were subjected to 30-min extinction sessions for 15 consecutive days. Responses at the lever activated the delivery mechanism but did not result in the delivery of alcohol.

Footshock stress-induced reinstatement

The day after the last extinction session, animals (n=36) were subjected to a footshock stress-induced reinstatement session. A 15-min electric (1.0 mA current intensity) footshock stress was delivered via the grid floor of the chamber under a variable-interval 40-s schedule (interval range: 10–70 s). We choose 1.0 mA because in a previous study (Hansson et al. 2006), we showed that in Wistar rats, the maximum level of responding is obtained at this current intensity. After termination of footshock, the levers were extended into the chambers, and responses were recorded for 30 min. Each group of animals (n=9/group) was tested for the effect of acute pre-treatment with URB597 (0.0, 0.1, 0.3 and 1.0 mg/kg) administered 30 min prior to the reinstatement session. The experiment was carried out using a between-subject design. Responding at the inactive lever was recorded throughout the experiment to monitor non-specific behavioural effects.

Yohimbine-induced reinstatement

The day after the last extinction session, animals (n=12) were injected with URB597 (0.0, 0.1, 0.3, 1.0 mg/kg) 30 min prior to administration of yohimbine (1.25 mg/kg).

After another 30 min, the reinstatement test was started. Here, URB597 was used with the purpose of inhibiting the effects of yohimbine, hence it was given 30 min prior to challenging the animals with the pharmacological stressor. Animals received all drug treatments according to a counterbalance Latin square design. A 3-day interval, during which animals were subjected to extinction sessions, was allowed between drug tests.

Alcohol-induced anxiety

To measure anxiety-like responses, the elevated plus-maze test was used. The apparatus consisted of two black wooden open arms and two enclosed arms (40 cm high walls), which were arranged so that the similar arms were opposite to each other. The maze, elevated 50 cm above the floor, was cleaned with water and dried after each trial. The 5-min test procedure began when the animal was placed in the centre of the maze, facing a closed arm. The percent of time spent in open arms and the percent of open arm entries were used as measures of anxiety-like behaviour, while the number of entries into the closed arms was used as an indicator of general motor activity (Cruz et al. 1994). As previously described by Gehlert et al. (2007), alcohol-induced anxiety was measured by treating rats (N=32) IP with 3.0 g/kg of 20% alcohol or vehicle (saline) and 12 h later running the elevated plus-maze (EPM) test. Rats (N=9/group) were treated with URB597 (0.3, 1.0 mg/kg) or its vehicle 30 min prior to the EPM. Another control group of rats (N=9) received alcohol and URB vehicles.

Statistics

Analysis of variance (ANOVA) of the results was followed by post hoc tests when appropriate. In particular, the effects of URB597 on alcohol self-administration, cue- and yohimbine-induced reinstatements were analysed by means of one-way ANOVA with repeated measures using drug dose as a within subject factor. Alcohol drinking in msP rats was studied by two-way ANOVA with one factor between (dose) and one factor within (time). Rimonabant effect was tested using one-way ANOVA within subject factor (drug dose) design. Statistical analysis of the footshock stress-induced reinstatement experiment was performed with one-factor ANOVA using the treatment as between-subject factor. For reinstatement experiments, differences among responses during the extinction and reinstatement sessions were analysed in the vehicle-treated group by one-way within-subjects ANOVA. The EPM test was evaluated using between-subject one-way ANOVA. Post hoc analysis was carried out using the Newman–Keuls test.

Results

Experiment 1: effect of URB597 on alcohol self-administration under FR1 and PR schedule of reinforcement

As shown in Table 1, Wistar rats acquired robust operant alcohol responding that was not modified by URB597 treatment. Responding at the inactive lever was very low and was not affected by drug treatment.

To confirm that URB597 did not affect motivation to self-administer alcohol, the highest dose of the compound was also tested under a PR schedule of reinforcement. Again, drug administration did not significantly modify animal behaviour, and the break point for alcohol in vehicle- and URB597-treated animals remained similar (12.3±4.5 and 10.7±3.5, respectively). Responses at the inactive lever were very low and were not affected by drug treatment (data not shown).

Experiment 2: effect of rimonabant on alcohol self-administration under FR1 schedule of reinforcement

As shown in Table 1, Wistar rats acquired robust operant alcohol responding that was significantly reduced [$F(3,9)$= 15.77; $p<0.01$] by rimonabant treatment. Responding at the inactive lever was very low and was not affected by drug treatment.

Table 1 Effect of IP treatment with the FAAH inhibitor URB597 or with the CB1 receptor antagonist rimonabant on FR1 10% alcohol self-administration in Wistar rats (n=8–10)

	URB597 (mg/kg)				Rimonabant (mg/kg)			
	0.0	0.1	0.3	1.0	0.0	0.3	1.0	3.0
Active lever	53.6±6.8	60.0±7.7	59.9±6.0	67.4±5.7	41.0±4.7**	23.3±5.7**	21.8±6.0**	14.1±3.1**
Inactive lever	2.6±0.7	4.1±1.3	4.1±1.7	5.6±2.1	2.0±0.9	1.6±0.8	3.6±2.8	1.3±0.7

Values represent the mean (±SEM) number of rewards earned at the active lever or responses at the nonreinforced inactive lever.
**$p<0.01$; significant difference from controls (0.0)

Experiment 3: effect of URB597 on two-bottle free choice alcohol drinking in msP rats

Previous studies showed that genetically selected alcohol-preferring msP rats are highly sensitive to cannabinoid receptor manipulation (Cippitelli et al. 2005), and therefore, we thought it interesting to extend the study of URB597 to these animals as well. As shown in Table 2, in less than 15 min, msP rats drank more than 1.0 g/kg of alcohol. Alcohol consumption was maintained for the entire test period (1 h), and treatment with URB597 did not significantly modify alcohol intake. Treatment with this FAAH inhibitor neither affected water intake nor significantly modified voluntary food consumption (Table 2).

Experiment 4: effect of URB597 on cue-induced reinstatement of alcohol seeking

Throughout the conditioning phase in which animals discriminated between alcohol or water availability, rats responded at a higher level for alcohol. ANOVA showed a significant overall effect of conditioning [$F(1,8)=21.210$; $p<0.01$]. On the last day of the discrimination period, animals reached a lever pressing response of 48.8±8.9 for alcohol, while the response for water was 26.6±7.0. During extinction, lever pressing progressively decreased and passed from 39.4±5.2 on the first day to 11.0±2.2 on the last extinction day. In the reinstatement test, the ANOVA showed that cues had a significant overall effect on alcohol seeking [$F(2,8)=32.290$; $p<0.001$; Fig. 1a]. A more detailed analysis showed a robust reinstatement of responding under the S^+/CS^+ [$F(1,8)=35.237$; $p<0.001$] but not under the S^-/CS^- compared with the last day of extinction. Reinstatement of alcohol-seeking behaviour was not significantly modified by pre-treatment with URB597. Responses at the inactive lever were not influenced by the treatment (Fig. 1b).

Experiment 5: effect of URB597 on footshock stress- and yohimbine-induced reinstatement of alcohol seeking

A stable baseline of responding for 10% (*v*/*v*) alcohol was established in 15 days. Lever presses on the last alcohol session were 41.8±4.3. During the extinction phase, responding progressively decreased and passed from 39.1±2.8 of the first day to 16.1±3.9 of the last extinction day. Exposure to footshock produced a marked increase in the number of lever presses in untreated animals [$F(1,8)=7.308$; $p<0.05$] in comparison to responses of the last extinction day. The ANOVA showed that pre-treatment with URB597 (0.1, 0.3, 1.0 mg/kg, IP) did not block footshock stress-induced reinstatement. Responding at the inactive lever was very low throughout the experiment and was not affected by shock or by treatment with URB597 (Fig. 2).

For yohimbine-induced reinstatement, after 15 days of operant responding for 10% (*v*/*v*) alcohol, animals established a baseline of 45.7±7.8 lever pressings. This response was extinguished in the subsequent 15 days, reaching values of 11.7±2.3. The intraperitoneal administration of the alpha-2 adrenoceptor antagonist yohimbine at the dose of 1.25 mg/kg significantly reinstated the operant response for alcohol [$F(1,11)=20.820$ $p<0.01$]. Pre-treatment with URB597 (0.1, 0.3, 1.0 mg/kg, IP) did not reverse yohimbine-induced alcohol-seeking behaviour [$F(3,11)=0.090$; NS]. Analysis of inactive lever responding revealed a marginal but significant effect of treatment with yohimbine on the inactive lever when it was compared with data of the last day of extinction [$F(1,11)=5.557$, $p<0.05$]. Treatment with URB597 did not significantly modify reinstatement at any dose tested (Fig. 3). Because of the

Table 2 Effect of the IP administration of the FAAH inhibitor URB597 (0.1, 0.3 and 1.0 mg/kg) or its vehicle (0.0) on cumulative alcohol and food intakes in ($n=9$) msP rats

Time (min)	URB 597 (mg/kg)			
	0.0	0.1	0.3	1.0
Alcohol intake (g/kg)				
15	1.0±0.1	1.1±0.1	1.1±0.1	1.2±0.2
30	1.2±0.1	1.3±0.1	1.2±0.1	1.3±0.2
60	1.3±0.1	1.4±0.2	1.5±0.1	1.5±0.1
Food intake (g/kg)				
15	1.0±0.7	1.3±0.7	0.7±0.4	0.3±0.2
30	1.3±0.7	2.2±0.9	1.9±0.7	1.1±0.8
60	3.2±1.5	4.7±1.1	5.0±0.8	5.5±2.4

Alcohol and water were offered in two-bottle free choice conditions with alcohol available for 60 min per day. Food pellets and water were always available. The intakes were monitored at 15, 30 and 60 min after exposure. Values represent the means (±SEM) of alcohol and food consumption expressed in g/kg.

effects of yohimbine on inactive lever responding, which is a potential measure of general (non-directed) activity and response generalization (Shalev et al. 2002), data were re-analysed using change-scores (active lever minus inactive lever presses). This change-score analysis replicated that of active lever responding [$F(1,11)=24.317$, $p<0.01$] for reinstatement and for the treatment, suggesting that inactive lever responding cannot account for the results obtained for active lever responding (Lê et al. 2005).

Experiment 5: effect of URB597 on alcohol-induced anxiety

Analysis of variance showed an overall significant EPM effect for both percent of time spent in the open arms and

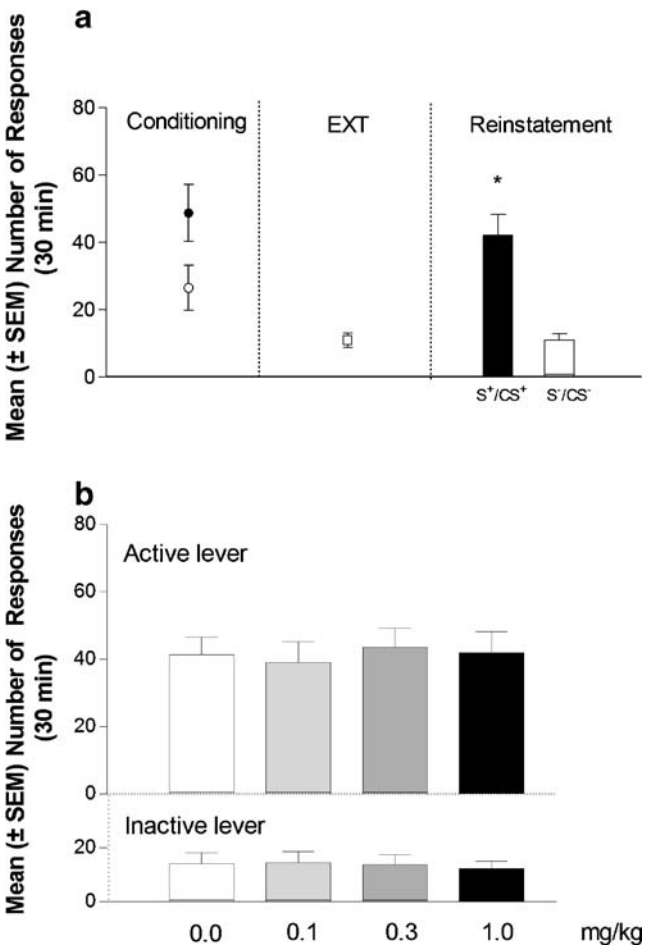

Fig. 1 **a** *Conditioning* Responses of the last 10% alcohol (*filled circle*) and water (*open circle*) session of the discrimination phase. *Extinction (EXT)* Responses during the last day of this phase. *Reinstatement* Responses in rats exposed to stimuli predictive of alcohol (S^+/CS^+) or water (S^-/CS^-) availability. Significant difference from *EXT*, $p<0.05$. **b** Effect of IP treatment with URB597 (0.1, 0.3 and 1.0 mg/kg) or its vehicle (0.0) on cue-induced reinstatement of alcohol seeking in ($n=9$) Wistar rats. Values represent the mean (±SEM) number of responses at the active or inactive levers. Significant difference from extinction, single asterisk $p<0.05$

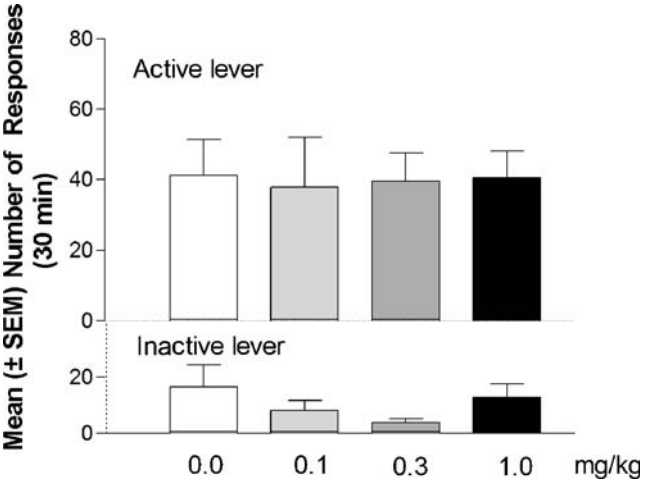

Fig. 2 Stress-induced reinstatement of alcohol seeking in ($n=36$) Wistar rats exposed for 15 min to intermittent electric footshock preceded by IP injection of URB597 (0.1, 0.3 and 1.0 mg/kg) or its vehicle (0.0). Compared to extinction, stress elicited a significant reinstatement of responding that was not affected by drug treatment. Values represent the mean (±SEM) number of responses at the active and inactive lever

percent of open arm entries ([$F(3,32)=16.65$ $p<0.001$ and [$F(3,32)=12.42$ $p<0.001$, respectively]. Post hoc analysis revealed that withdrawal from a single large dose of alcohol elicits a marked anxiogenic response, resulting in a significant reduction in percent of time spent in the open arms ($p<0.01$) and open arms entries ($p<0.01$). Treatment with URB597 completely abolished the anxiogenic-like effects of alcohol, and, as shown in Fig. 4, anxiety was completely reversed at both drug doses (0.3 and 1.0 mg/kg)

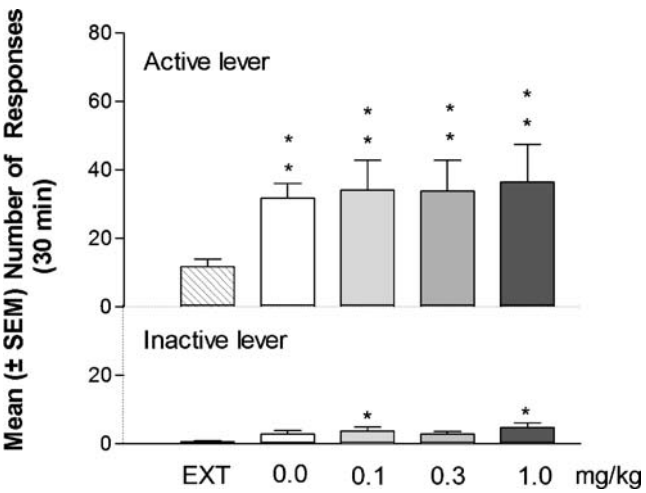

Fig. 3 Yohimbine-induced reinstatement of alcohol seeking in ($n=12$) Wistar rats previously treated with URB597 (0.1, 0.3 and 1.0 mg/kg) or its vehicle (0.0). Compared to extinction (*EXT*), yohimbine elicited a significant reinstatement of responding that was not affected by drug treatment. Values represent the mean (±SEM) number of responses at the active and inactive lever. Significant difference from extinction, *double asterisk* $p<0.01$ and *single asterisk* $p<0.05$

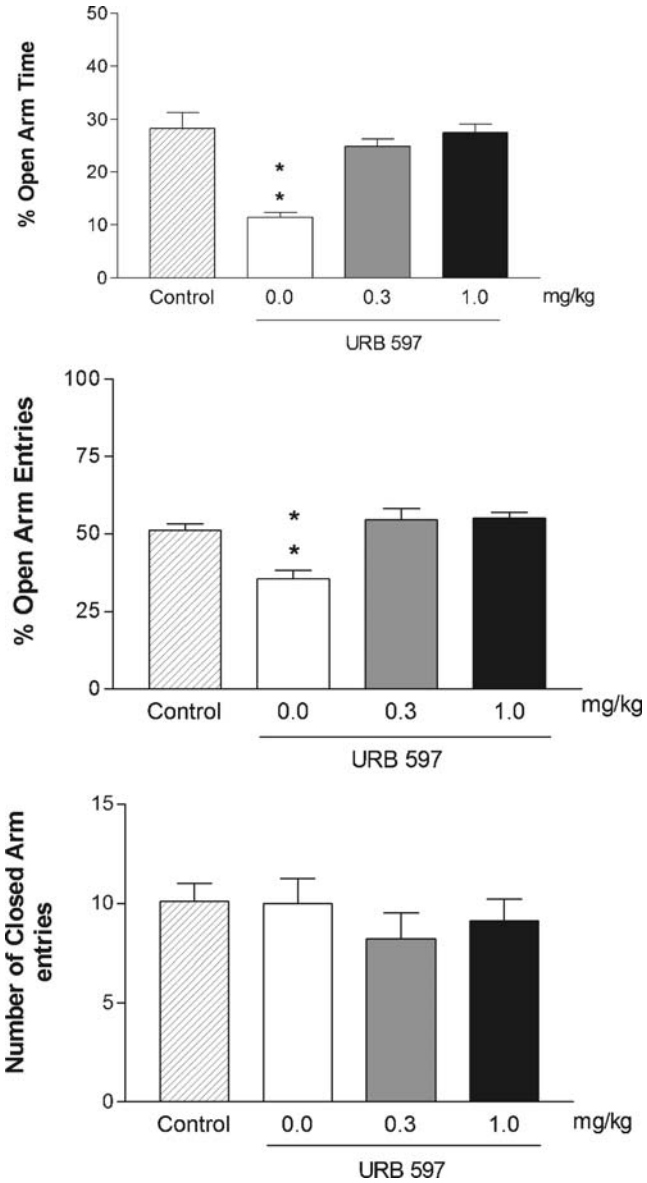

Fig. 4 Effect of IP treatment with URB597 (0.3 and 1.0 mg/kg) or its vehicle (0.0) on animals treated with a single dose of 3.0 g/kg of 20% alcohol given IP 12 h prior to EPM test. **a** Percent of time spent in open arms. **b** Percent of open arms entries. **c** Number of closed arms entries. Control group received alcohol and URB597 vehicles. Significant difference from control, *double asterisk* $p<0.01$

tested. Closed-arm entries were not affected by alcohol administration nor were they modified by URB597 treatment [$F(3,32)=0.59$ NS]. This finding rules out the possibility that results were influenced by non-specific alteration of the animals' locomotor activity.

Discussion

Results showed that under our experimental condition treatment with URB597, a potent, selective and systemically active inhibitor of FAAH (Kathuria et al. 2003), the enzyme responsible for the inactivation of the endocannabinoid anandamide did not affect operant alcohol self-administration in Wistar rats nor did it modify the break point for alcohol. In a previous study (Ciccocioppo et al. 2006), we showed that compared to nonselected Wistar controls, naïve msP rats have greater cannabinoid CB1 receptor mRNA levels in a number of brain areas including the frontoparietal cortex, caudate–putamen, and hippocampus. In these animals, CB1 receptor mRNA expression returns to the same levels of Wistar controls after voluntary alcohol consumption (Cippitelli et al. 2005). These findings prompted us to hypothesise that alcohol consumption in msP rats could be partly linked to an overfunctioning endocannabinoid system and possibly that these animals could have different sensitivity to manipulation of this system, reflected in their alcohol drinking. Hence, we tested the effects of URB597 on homecage drinking in these animals as well. Once again, results demonstrated that FAAH inhibition does not change alcohol consumption, thus extending our finding to animals with innate propensity to prefer alcohol. In msP rats, a modest trend of increased alcohol consumption was observed at the highest URB597 dose, but an identical trend was observed for food consumption, suggesting that increased brain anandamide levels may stimulate appetitive behaviour in general. This is in line with the very well characterised stimulatory effects on food intake following CB1 receptor activation (Williams et al. 1998; Gomez et al. 2002).

Previous reports showed that administration of direct cannabinoid receptor agonists like CP 55,940 and WIN 55,212-2 increases alcohol intake and alcohol reinforcement in both nonselected and genetically selected alcohol-preferring rats (Colombo et al. 2002; Gallate et al. 1999). These effects are mediated by activation of CB1 receptors because they are abolished by pre-treatment with the selective CB1 receptor antagonist rimonabant. These data contrast with results obtained here with URB597. We speculate, therefore, that increase of brain anandamide levels by modulation of endocannabinoid tone evokes alcohol-drinking effects substantially different from those reported after administration of CB1 receptor direct agonists. Consistent with this finding, previous reports showed that FAAH inhibitors may have distinct pharmacological properties compared to direct agonists at CB1 receptors. For example, it was shown that WIN 55,212-2 and CP 55,940 produce a characteristic combination of four Δ^9-THC-like symptoms: hypothermia, analgesia, hypoactivity, and catalepsy, all of which are reversed by rimonabant (Chaperon and Thiebot 1999, Piomelli et al. 2006). Conversely, hypothermia, analgesia and catalepsy induced by anandamide are not reversed by SR 141716 (Chaperon and Thiebot 1999). In addition, it was shown

that the systemic dose of 0.3 mg/kg of URB597 that maximally blocks FAAH activity does not mimic exogenous anandamide in producing catalepsy, hypothermia or hyperphagia (Kathuria et al. 2003). Further differences in the profiles of direct and indirect cannabinoid receptor agonists were seen by Cippitelli et al. (2007) who found that AM404, an anandamide uptake blocker, reduces alcohol self-administration, does not potentiate alcohol-induced hypothermia and hypolocomotion and does not affect cue-induced reinstatement of alcohol seeking. AM404 reduction of alcohol self-administration was not dependent on CB1, CB2, or vanilloid VR1 receptors, suggesting alternative targets for its pharmacological actions (Cippitelli et al. 2007).

In a recent report, Vinod et al. (2006) reported reduction in FAAH activity and increased cortical levels of anandamide in mice exposed to alcohol vapour for 72 h. Moreover, Hansson et al. (2007) found reduced FAAH expression and activity in the prefrontal cortex of Finnish alcohol-preferring AA rats when compared with ANA. The same study showed that intra-prefrontal cortex administration of URB597 increased operant alcohol self-administration in nonselected Wistars. More important, Blednov et al. (2007) reported that FAAH knockout mice voluntarily consume more alcohol than wild-type littermates, while treatment with URB597 increases alcohol intake in wild-type mice. In contrast with these earlier findings, in the present study, alcohol intake was not affected by URB597 administration. However, at the same doses tested on alcohol drinking, we showed that this FAAH inhibitor reverses alcohol-induced anxiety. This demonstrates, therefore, that under our experimental conditions, URB597 was used at doses that are able to control behavioural responses associated to alcohol. Moreover, data showing that rimonabant reduced alcohol self-administration demonstrate that the experimental condition under which our study was carried out are appropriate to detect inhibitory effects on ethanol drinking. At present, the reasons for the discrepancy between the results of the present work and the previous studies are unclear, though it is possible that several methodological factors may be involved. For example, Hansson et al. (2007) microinjected the compound directly into the prefrontal cortex, thus selectively increasing endocannabinoid activity in this nucleus. In our experiments, URB597 was injected peripherally at doses that can inhibit FAAH activity in the whole brain (Kathuria et al. 2003). Several major methodological differences can be also identified between our study and the report published by Blednov et al. (2007). For example, our experiments used Wistar rats operantly self-administering alcohol or msP rats taking alcohol under limited access conditions. Blenov and colleagues used mice, and alcohol was available daily. There are also studies showing that environmental stress can markedly affect endocannabinoid neurotransmission (Hill et al. 2006; Patel et al. 2005; Rademacher and Hillard 2007), and thus it can be speculated that different responses to the blockade of FAAH may also depend on the endogenous tone of the system at the moment of drug administration.

Alcoholism is a chronic relapsing disorder characterised by high recidivism rates. Two major factors triggering relapse behaviour are stress and environmental conditioning experiences (American Psychiatric Association 1994; O'Brien and McLellan 1996; O'Brien et al. 1990, 1998), which probably facilitate relapse to alcohol seeking via distinct brain mechanisms. For example, activation of the mesolimbic dopamine system via an opioid-dependent mechanism (or via direct alterations in dopamine transmission in the basolateral nucleus of amygdala) seems to mediate the effect of drug-associated cues (Liu and Wiess 2002; Ciccocioppo et al. 2001), and extrahypothalamic CRF within the bed nucleus of the stria terminalis and median raphe nucleus is likely to mediate stress-induced reinstatement of drug-seeking behaviour (Shaham et al. 2000; Lê and Shaham 2002).

Various studies have observed involvement of the cannabinoid system in the regulation of relapse behaviour. For example, antagonism at the CB1 receptor attenuates cue-induced reinstatement to alcohol seeking in both Wistar and genetically selected alcohol-preferring rats (Cippitelli et al. 2005, Economidou et al. 2006). Conversely, treatment with CB1 receptor antagonist fails to prevent footshock stress-induced alcohol relapse (Economidou et al. 2006). Similar effects were described in animals trained to self-administer cocaine and then tested for stress or cue-induced reinstatement (De Vries et al. 2001; De Vries et al. 2003; McGregor et al. 2005). Moreover, Lopez-Moreno and colleagues showed that administration of the CB1 receptor agonist WIN 55,212-2 during abstinence elicits a prolonged increase in alcohol drinking once alcohol is made available again. To shed some light on the significance of the endocannabinoid system in the regulation of relapse-like behaviour, therefore, we also investigated the effects of URB597 on alcohol seeking elicited by conditioning factors and stress. Consistent with our alcohol drinking data, URB597 at doses (0.3–1.0 mg/kg) that completely block FAAH did not affect relapse to alcohol seeking induced by either cues or stress. These data further confirm the absence of a primary role of anandamide in the regulation of alcohol-ingestive behaviours in the rat.

Several reports indicate that in rodents, increases of brain anandamide levels following reuptake inhibition or metabolic degradation result in a significant anxiolytic-like response and analgesia (Bortolato et al. 2006; Kathuria et al. 2003; Hohmann et al. 2005; Piomelli et al. 2006). Consistent with this finding, our results show that URB597

prevents anxiety-like responses elicited by an acute IP injection of a high alcohol dose. Based on these pharmacological actions, one could have also expected to observe reduction in footshock stress- or yohimbine-induced reinstatement of alcohol seeking after URB597 administration. However, the data did not support this prediction.

In conclusion, our results showed that administration of the FAAH inhibitor URB597 did not affect intake or motivation to drink alcohol nor did it alter alcohol-seeking behaviour. Our findings neither confirmed nor supported the hypothesis that increased anandamide levels in the brain contribute to sustain high alcohol drinking or facilitate relapse to alcohol seeking. We speculate that drugs like URB597 or other endocannabinoid modulators may offer important advantages over drugs that activate CB1 receptors directly. For instance, CB1 receptor direct agonists show intrinsic abuse potential and increase consumption of other drugs of abuse (Colombo et al. 2002; Gallate et al. 1999; McGregor et al. 2005). Conversely, as shown here, modulation of endogenous anandamide levels did not increase alcohol abuse-related behaviours. In the present investigation, we have also demonstrated that even though URB597 possesses potent anxiolytic-like properties, it fails to prevent footshock- and yohimbine-induced reinstatement to alcohol seeking. This finding is significant in that it supports the validity of these widely used animal models of relapse, demonstrating that these models are not sensitive to general manipulation of anxiety-related behaviour.

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