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# The anandamide transport inhibitor *AM404* reduces ethanol self-administration

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## Abstract

The endocannabinoid system mediates in the pharmacological actions of ethanol and genetic studies link endocannabinoid signaling to alcoholism. Drugs activating cannabinoid CB1 receptors have been found to promote alcohol consumption but their effects on self-administration of alcohol are less clear because of the interference with motor performance. To avoid this problem, a novel pharmacological approach to the study of the contribution of the cannabinoid system in alcoholism may be to use drugs that locally amplify the effects of alcohol on endogenous cannabinoids. In the present study we addressed this model by studying the effects of the anandamide transport inhibitor *N*-(4-hydroxyphenyl) arachidonoyl-ethanolamide (*AM404*) on both ethanol self-administration and reinstatement of alcohol-seeking behavior in rats. The results show that *AM404* significantly reduced ethanol self-administration in a dose-dependent manner but failed to modify reinstatement for lever pressing induced by the stimulus associated with alcohol. This effect was not due to a motor depressant effect and was not related to a decrease in general motivational state, as it was not effective in other reward paradigms such as lever pressing for a saccharin solution. The mechanism of action of *AM404* does not involve cannabinoid CB1 receptors as the CB1-selective antagonist SR141716A did not block the reduction of ethanol self-administration induced by the anandamide uptake blocker. Moreover, 3-(1,1-dimethylheptyl)-(-)-11-hydroxy- $\Delta^8$ -tetrahydrocannabinol (HU-210), a classical cannabinoid receptor agonist, did not affect ethanol self-administration. The effects of *AM404* are not mediated by either vanilloid VR1 receptors or cannabinoid CB2 receptors because it is not antagonized by either the VR1 receptor antagonist capsazepine or the CB2 antagonist AM630. These results indicate that *AM404* may be considered as an innovative approach to reduce alcohol consumption.

## Introduction

Cannabis and alcohol are two of the oldest drugs used by humans. Together with nicotine, they represent a relevant health problem because of the clinical consequences of their abuse. Their psychotropic effects are well known and recent research has shown that there is a close link between cannabis and alcohol (Arnone *et al.*, 1997; Basavarajappa & Hungund, 2002). The endogenous cannabinoid system [a functional set of lipid transmitters and receptors that is the target of both natural and synthetic cannabinoids (Piomelli, 2003)] has been shown to mediate some of the pharmacological and behavioral aspects of alcohol (Basavarajappa & Hungund, 2002; Rodríguez de Fonseca *et al.*, 2005; Ferrer *et al.*, 2007). Both cannabinoids and alcohol activate the same reward pathways and the cannabinoid CB1 receptor plays an important role in regulating the positive reinforcing effects of alcohol as well as alcohol relapse (Cippitelli *et al.*, 2005; Hungund *et al.*, 2003; Wang *et al.*, 2003).

Several studies have documented that endocannabinoid transmission becomes hyperactive in reward-related areas during chronic ethanol administration. This hypothesis is based on two findings. First, the increase in the levels of both anandamide and 2-arachidonylglycerol, the two main endocannabinoids, observed in animals chronically exposed to ethanol. Second, the down-regulation of CB1 receptors induced by endocannabinoid-mediated over-stimulation (Basavarajappa & Hungund, 2002). Following this rationale, cannabinoid CB1 receptor knockout mice show reduced alcohol preference and self-administration (Hungund *et al.*, 2003; Poncelet *et al.*, 2003; Wang *et al.*, 2003; Naassila *et al.*, 2004).

However, the role of cannabinoids in alcohol- and drug-induced reward modulation demands further research because of the few studies addressing the actions of cannabinoid CB1 receptors on self-administration paradigms, with respect to the more extensive research performed in alcohol consumption tests. Cannabinoid CB1 receptor agonists increase ethanol consumption in normal and alcohol-prefering/alcohol-avoiding rodent strains (Colombo *et al.*, 2002; Kelai *et al.*, 2006). The involvement of cannabinoid CB1 receptors is further supported by the effects of the cannabinoid receptor antagonist SR141716A, which reduces ethanol intake and preference (Arnone

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*et al.*, 1997; Colombo *et al.*, 1998; Rodriguez de Fonseca *et al.*, 1999; Rinaldi-Carmona *et al.*, 2004; Cippitelli *et al.*, 2005). This modulatory action of cannabinoid CB1 receptors in the reinforcing/rewarding effects of ethanol is further indicated by the reduction of ethanol-rewarding properties in cannabinoid CB1 receptor knockout mice (Naassila *et al.*, 2004; Hungund *et al.*, 2003; Wang *et al.*, 2003; Houchi *et al.*, 2005).

Although the activation of cannabinoid CB1 receptor increases ethanol consumption, there are no clear indications that it may result in enhanced ethanol self-administration or relapse (McGregor *et al.*, 2005). Moreover, studies suggest that activation of CB1 receptor reduces operant responding for drugs and food in a variety of paradigms (Jarbe *et al.*, 2003; Drews *et al.*, 2005). As an example, cannabinoid CB1 receptor agonists decrease both cocaine intravenous self-administration in rats (Fattore *et al.*, 1999) and the reinforcing actions of cocaine (Vlachou *et al.*, 2003); another study by Braidia & Sala (2002) confirmed that the combination of the cannabinoid CB1 receptor agonist CP55 940 with methylene dioxy methylamphetamine reduced the number of drug-associated lever pressings. However, the cannabinoid CB1 receptor antagonist SR141716A has been shown to block the rewarding properties of both food and most drugs of abuse. The treatment with SR141716A reduced both the self-administration and the subjective effects of tetrahydrocannabinol in rats, monkeys and humans (Tanda *et al.*, 2000; Huestis *et al.*, 2001; Solinas *et al.*, 2003). It also decreased heroin self-administration (De Vries *et al.*, 2003; Navarro *et al.*, 2001; Solinas *et al.*, 2003) as well as morphine-induced conditioned place preference (Chaperon *et al.*, 1998; Martin *et al.*, 2000), nicotine self-administration (Cohen *et al.*, 2002) and nicotine-induced conditioned place preference (Le Foll & Goldberg, 2004). In at least one study, cannabinoid receptor antagonism produces a biphasic effect, with a transient increase in heroin self-administration followed by a profound inhibition of operant responding for the opiate (Navarro *et al.*, 2001).

The different effects of cannabinoid receptor agonists on alcohol consumption vs. alcohol self-administration have been interpreted as being due to differences in apparatus, experimental design and subjects used. To date, although there is a consensus in the literature with regard to the ability of cannabinoids to increase ethanol consumption, we need to clarify the role of cannabinoid receptors in operant responding for ethanol. This is important in order to establish the contribution of the endogenous cannabinoid system in alcoholism and may help to design new endocannabinoid-based therapies for this common addiction. The wide distribution of cannabinoid receptors and their role as a modulator of synaptic transmission make difficult the interpretation of these actions of direct cannabinoid receptor agonists/antagonists on alcoholism. They also limit the utility of direct cannabinoid CB1 receptor ligands for the treatment of drug abuse. A pharmacological alternative that might reduce these problems may be offered by anandamide reuptake inhibitors. These drugs have been used *in vivo* in an effort to demonstrate their ability to inhibit cellular accumulation of anandamide and thereby stimulate cannabimimetic signaling. The anandamide reuptake inhibitor *N*-(4-hydroxyphenyl) arachidonoyl-ethanolamide (AM404) produces physiological effects similar to anandamide *in vivo* and potentiates the receptor-mediated effects of exogenously administered anandamide (Beltramo *et al.*, 1997, 2000; Calignano *et al.*, 1997; Giuffrida *et al.*, 2000). Although numerous studies have examined and compared the pharmacology of cannabinoid agonists and antagonists in reinforcing effects of ethanol, there are no studies addressing the effects of the anandamide transport blocker AM404 on alcohol-seeking behaviors (ethanol self-administration and relapse). Considering the importance

of the endocannabinoid system in ethanol intake and reward, as well as the dynamic changes in anandamide production during acute and chronic ethanol exposure (Basavarajappa & Hungund, 2002; Ferrer *et al.*, 2007), we decided to study both the effects of the administration of the anandamide reuptake blocker AM404 in rats trained to self-administer ethanol and its actions in rats exposed to relapse induced by contextual stimuli previously associated with ethanol.

## Materials and methods

### Animals

Male Wistar rats weighing 175–225 g were housed in groups of two in a temperature- and humidity-controlled vivarium on a reverse 12-h light/dark cycle (lights on 18:00 h; off 06:00 h). All training and experimental sessions were conducted during the dark phase of the cycle. Standard laboratory rat chow and water were available *ad libitum* in the home cage, except as noted below. All experimental procedures met the guidelines for the care and use of laboratory animals of the European Communities directive 86/609/EEC regulating animal research and were approved by the ethical committee of Carlos Haya Hospital, Malaga (Spain).

### Drugs

The anandamide transport inhibitor AM404, cannabinoid CB1 receptor selective agonist ACEA (Sigma, Italy), non-selective cannabinoid receptor agonists WIN 55,212-2 and 3-(1,1-dimethylheptyl)-(-)-11-hydroxy-delta 8-tetrahydrocannabinol (HU-210) (Biogen Tocris, UK), vanilloid receptor antagonist capsazepine (Sigma, Italy) and cannabinoid CB2 receptor antagonist AM630 (Biogen Tocris, UK) were mixed in a vehicle of dimethylsulfoxide, Tween 80 and distilled water in a ratio of 10 : 10 : 80 (v : v). They were injected intraperitoneally 30 min prior to the self-administration session at a volume of 1 mL/kg at doses of 0.4, 2 and 10 mg/kg (AM404); 0.2, 1 and 2 mg/kg (ACEA); 0.4, 2 and 5 mg/kg (WIN 55,212-2); 4, 10 and 20 µg/kg (HU-210); 3 and 10 mg/kg (capsazepine); and 1.25 and 2.5 mg/kg (AM630). The selective CB1 receptor antagonist SR141716A (Sanofi-Synthelabo, Montpellier, France) was suspended in two to three drops of Tween 80 in saline as vehicle and was administered intraperitoneally at doses of 0.1 and 1 mg/kg. Pre-injection times were based upon previous work with these drugs in our laboratory and/or surveys of the scientific literature.

### Locomotor studies

We studied the effects of AM404 (0.4, 2 and 10 mg/kg, *i.p.*), ACEA (0.2, 1 and 2 mg/kg, *i.p.*), WIN 55,212-2 (0.4, 2 and 5 mg/kg, *i.p.*) or vehicle on immobility and horizontal locomotor activity in Wistar rats ( $n = 8–10$ /group). Motor activity was studied in an open field (100 × 100 cm, divided into 25 20 × 20 cm squares) interfaced to a computer (SMART, Panlab, Barcelona, Spain) that recorded activity automatically. Animals were placed in the arena for 10 min the day before testing for habituation. On the experimental day, the animals were placed in the center of the testing chambers, and immobility (time spent by the animal in absolute quietness) and horizontal activity (number of squares crossed by the animal in a given sample period) were recorded at 5 min intervals. This procedure was performed at 0, 30, 60 and 120 min after the injection of either vehicle or each compound. All behavioral tests were conducted in a sound-isolated room,

illuminated with an indirect halogen light (125 lx). Testing arenas were thoroughly cleaned between subjects.

#### *Effects of the combination of ethanol and AM404 on locomotion and hypothermia*

Both cannabinoids and alcohol lower body temperature and depress locomotor activity. In order to assess the potential additive effects of ethanol and AM404 on hypothermia and hypolocomotion, we studied the effects of the combined treatment with AM404 (2 mg/kg, i.p.) 30 min before ethanol injection (2 g/kg, 20% in saline, i.p.). Motor activity was studied in an open field as described above in addition to body temperature, which was measured with a rectal probe. This procedure was performed at 0, 30, 60 and 120 min after the injection of either vehicle or AM404 ( $n = 8-10$ /group). All procedures were conducted in a sound-isolated room, illuminated with an indirect halogen light (125 lx).

#### *Operant training for liquid reinforcers*

Training and testing were conducted in standard operant chambers located in sound-attenuating, ventilated environmental cubicles. Each chamber was equipped with a drinking reservoir (capacity 0.10 mL), positioned 4 cm above the grid floor in the center of the front panel of the chamber, and a retractable lever, located 3 cm to the right 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 behavioral data. Rats were trained to self-administer 10% ethanol (v/v), 0.2% saccharin (w/v) or water in 30 min daily sessions on a fixed-ratio 1 schedule of reinforcement, where each response resulted in delivery of 0.1 mL of fluid as previously described (Weiss *et al.*, 1993). Briefly, for the first 3 days of training, water availability in the home cage was restricted to 2 h/day in order to facilitate acquisition of operant responding for a liquid reinforcer. During this time, rats were permitted to lever-press for a 0.2% (w/v) saccharin solution. At this point, water was made freely available and saccharin self-administration training continued for another 3 days. The rats were then trained to self-administer ethanol by using a modification of the sucrose-fading procedure (Samson, 1986) that used saccharin instead of sucrose (Weiss *et al.*, 1993). During the first 6 days of training rats were allowed to lever-press for a 5.0% (w/v) ethanol solution containing 0.2% saccharin (w/v). Starting on day 7, the concentration of ethanol 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%. At the beginning of the saccharin-fading procedure a second but inactive lever was introduced. Responses at this lever were recorded during all training and testing phases as a measure of non-specific behavioral activation but they had no programmed consequences.

#### *Saccharine self-administration: effect of AM404*

Following completion of the saccharin training, the rats were used to study the effects of the administration of vehicle or AM404 (0.4 and 2 mg/kg) given 30 min prior to the self-administration session. The experiment was conducted every fourth day using a Latin square counterbalanced design. Responding at the inactive lever was recorded throughout the experiment to monitor non-specific behavioral effects.

#### *Ethanol self-administration. Effects of AM404 alone or in combination with SR141716A, AM630 or capsazepine and effects of the cannabinoid CB1 receptor agonists ACEA, WIN 55,212-2 and HU-210*

Following completion of the saccharin-fading procedure, the rats were trained in a 30 min session/day to lever-press for 10% ethanol until a stable baseline of responding was reached. In the first experiment, we studied the effect of the anandamide transport inhibitor AM404 (0.4, 2 and 10 mg/kg). In the second experiment, we pre-treated the animals either with the selective CB1 antagonist SR141716A (0.1 and 1 mg/kg), the cannabinoid CB2 receptor antagonist AM630 (1.25 and 2.5 mg/kg) or the vanilloid receptor VR1 competitive antagonist capsazepine (3 mg/kg) prior to the injection of AM404 (2 mg/kg). In the third experiment we studied the effects of the selective CB1 agonist ACEA (0.2, 1 and 2 mg/kg) or the non-selective CB1 receptor agonists WIN 55,212-2 (0.4, 2 and 5 mg/kg) or HU-210 (4, 10 and 20  $\mu$ g/kg) given 30 min prior to self-administration session. Pre-treatments were given 30 min prior to AM404 injection. The experiments were conducted every fourth day using a Latin square counterbalanced design using  $n = 8-10$  animals for each different experiment performed. Responding at the inactive lever was recorded throughout the experiment to monitor non-specific behavioral effects.

#### *Progressive ratio schedule of reinforcement: effect of AM404*

In this experiment, rats ( $n = 8$ ) were tested under a progressive ratio schedule of reinforcement to measure the break point (the last ratio completed by the animals) for ethanol. For this purpose, animals were first trained to self-administer 10% alcohol under a fixed ratio 1 schedule of reinforcement (see above). Following the acquisition of a stable baseline of responding for 10% ethanol, rats were tested under the progressive ratio condition, in which the response requirement (i.e. the number of lever responses or the ratio required to receive one dose of 10% ethanol) was increased as follows. For each of the first four ethanol deliveries the ratio was increased by 1; for the next four deliveries the ratio was increased by 2 and for all of the following deliveries the ratio was increased by 4. Each ethanol-reinforced response resulted in a 1.0 s illumination of the house light, whereas sessions were terminated when more than 30 min had elapsed since the last reinforced response. Drug testing was carried out once a week as follows. The progressive ratio baseline was established on days 1 and 2, whereas progressive ratio drug testing took place on day 3. For the next 2 days, animals were placed in the chambers under fixed ratio 1 condition to re-establish the ethanol self-administration baseline, whereas on days 6 and 7 they remained confined to their home cages. AM404 (0.4 and 2.0 mg/kg, i.p.) or its vehicle was given 30 min before the progressive ratio session. The experiment was repeated for the following 2 weeks, counterbalancing the treatment.

#### *Reinstatement of ethanol-seeking behavior: effect of AM404*

##### *Conditioning phase*

At completion of the fading procedure, animals were trained to discriminate between 10% ethanol and water in 30 min daily sessions. Beginning with self-administration training at the 10% ethanol concentration, discriminative stimuli predictive of ethanol vs. water availability were presented during the ethanol and water self-administration sessions, respectively. The discriminative stimulus for ethanol consisted of the odour of an orange extract (S+), whereas water availability (i.e. no reward) was signaled 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 ethanol 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 ethanol 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. The rats were only given ethanol sessions during the first 3 days of the conditioning phase. Subsequently ethanol and water sessions were conducted in random order across training days, with the constraint that all rats received a total of 10 ethanol and 10 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 discriminative stimulus. 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. These tests 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 ethanol S+ or water S- paired stimuli. The respective discriminative stimulus remained present during the entire session and responses at the previously active lever were followed by activation of the delivery mechanism and a 5 s presentation of the CS+ in the S+ condition or the CS- (tone) in the S- condition. Animals ( $n = 8$ ) 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 and 14), in which AM404 was administered 30 min prior to the sessions. Responding at the inactive lever was constantly recorded to monitor possible non-specific behavioral effects.

#### *Statistics*

Statistics were assessed by ANOVA. Effects regarding ethanol and saccharin self-administration, progressive ratio and reinstatement experiments were analysed with one-way ANOVA with repeated measures using drug treatment as a within-subject factor. Data related to locomotor activity and hypothermia were analysed with two-way ANOVA using the treatment as a between-subject factor and different time points as a within-subject factor. In the presence of overall significant effects ( $P$ -values  $< 0.05$ ), the Student-Newman-Keuls' post-hoc test was performed.

## **Results**

### *Experiment 1: AM404 decreases ethanol operant responses for ethanol but not saccharin*

Pre-treatment with the anandamide transport inhibitor AM404 30 min prior to the ethanol self-administration session significantly reduced the operant response for ethanol in a dose-dependent manner

(Fig. 1A). This effect was not due to a decrease in the reinforcing value of ethanol because progressive ratio experiments resulted in similar break points for animals treated with vehicle or AM404 (Fig. 1B). They were not derived or a motor inhibition induced by AM404 as the 2 mg/kg dose did not affect locomotion at the time of operant behavior testing (intervals 30 and 60 min, Fig. 1C). The effects were selective for ethanol because pre-treatment with AM404 did not modify operant responding for saccharin (0.2%, Fig. 1D). In addition, administration of AM404 did not alter food motivation and thus, food intake in rats deprived of food for 24 h (data not shown). These results suggest that the pharmacological effects of the anandamide transport inhibitor are not related to a devaluation of the motivational state or a devaluation of motivational properties of natural reinforcers.

### *Experiment 2: AM404 did not potentiate motor inhibition and hypothermia induced by acute ethanol administration*

Pre-treatment with AM404 (2 mg/kg) did not potentiate the hypolocomotion induced by ethanol (2 g/kg) during an extended period of analysis (Fig. 2A). Although AM404 induces a small decrease in body temperature, the combination with ethanol did not result in enhanced hypothermia (Fig. 2B). These results further indicate that the decrease in ethanol self-administration observed after pre-treatment with AM404 is not derived from a potentiation of the sedative actions of ethanol.

### *Experiment 3: lack of action of AM404 on ethanol-seeking behavior*

In a subsequent experiment, we tested the efficacy of AM404 as a modulator of not only the operant responses for ethanol but also the operant responses elicited by the contextual stimuli associated with alcohol. As the highest dose tested (10 mg/kg) resulted in significant inhibition of locomotion, we did not administer it in this context. Once a stable extinction baseline was observed, we induced relapse by presenting cues associated with ethanol delivery during training. Ethanol-related contextual stimuli elicited ethanol-seeking behavior, as operant responses induced by ethanol-associated stimuli were more intense and significantly higher than those observed on the last day of extinction (Fig. 3A). When AM404 was injected 30 min prior to cue presentation, it failed to alter the responses for ethanol seeking (Fig. 3B), indicating that anandamide uptake inhibition was not effective in preventing cue-induced relapse.

### *Experiment 4: the effects of AM404 are not reversed by cannabinoid CB1 receptor antagonism, cannabinoid CB2 receptor blockade or vanilloid VR1 receptor antagonism*

Pre-treatment with the cannabinoid receptor antagonist SR141716A (1 mg/kg) did not reverse AM404-induced reduction of operant responding for ethanol (Fig. 4A). However, as this compound reduces ethanol self-administration *per se*, it is plausible to consider that we may not observe such antagonism. Even when using a low dose of the antagonist (0.1 mg/kg) the trend of lever responding decreased and we failed to observe the reversal of actions of AM404. Operant responses after the different treatments were as follows: (i) vehicle-treated,  $44.9 \pm 10.7$ ; (ii) AM404 (2 mg/kg),  $27.0 \pm 5.4$  ( $P < 0.05$  vs. vehicle-treated animals); (iii) SR141716A (0.1 mg/kg),  $29.4 \pm 3.6$ ; and (iv) SR141716A (0.1 mg/kg) + AM404 (2 mg/kg),  $27.3 \pm 7.6$  ( $P < 0.05$  vs. vehicle-treated animals).

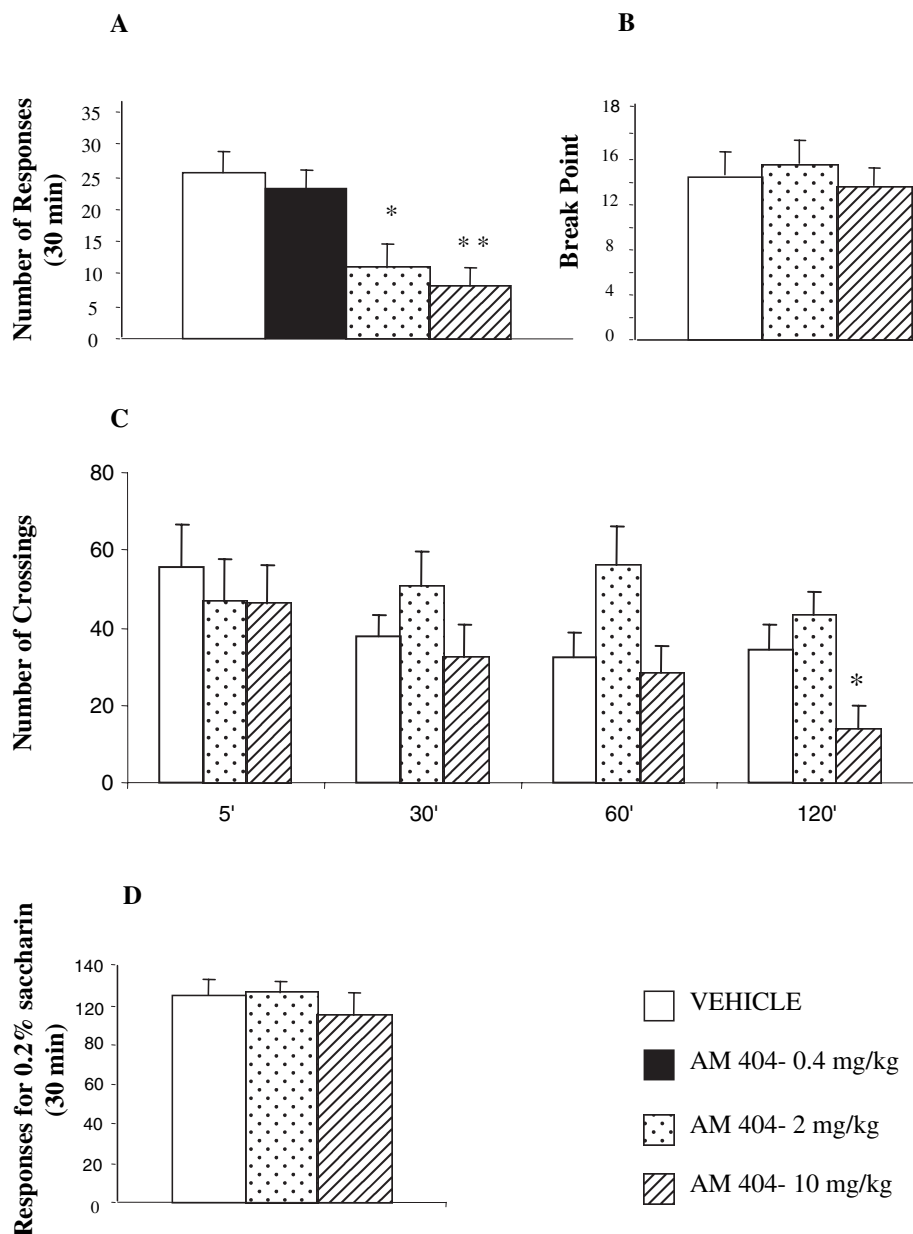


FIG. 1. Acute administration of the anandamide transport AM404 inhibitor (0.4, 2 and 10 mg/kg, i.p.) 30 min before the testing procedure in (A) ethanol self-administration; (B) progressive ratio for ethanol; (C) locomotion in the open field and (D) saccharin (0.2%) self-administration. Data are means  $\pm$  SEM of 8–10 determinations/group. \* $P < 0.05$  vs. vehicle-treated animals. \*\* $P < 0.01$  vs. vehicle-treated animals.

We then tested the hypothesis of a potential role of cannabinoid CB2 receptors in the mediation of AM404 responses, as CB2 receptors have been recently described in the brain, in neuronal circuits related to reward and learning (Van Sickle *et al.*, 2005). Pre-treatment with AM630, a cannabinoid CB2 receptor blocker, did not modify operant responses for alcohol at both doses used (data not shown) or counteract the inhibitory effects of AM404. Operant responses after the different treatments were as follows: (i) vehicle-treated,  $46.9 \pm 4.6$ ; (ii) AM404 (2 mg/kg),  $27.1 \pm 4.4$  ( $P < 0.05$  vs. vehicle-treated animals); (iii) AM630 (2.5 mg/kg),  $46.9 \pm 3.1$ ; and (iv) AM630 + AM404,  $23.0 \pm 5.1$  ( $P < 0.05$  vs. vehicle-treated animals).

Finally, we tested another target of both anandamide and AM404, a ligand-activated cation channel called vanilloid receptor VR1. To

analyse whether this receptor is involved in the actions of AM404 we first studied whether the VR1 antagonist capsazepine affected ethanol self-administration. As shown in Fig. 4B, all of the doses tested resulted in a significant alteration in operant responses for ethanol. Moreover, when administered prior to AM404, the competitive VR1 antagonist capsazepine (3 mg/kg) did not counteract the effect of AM404 (Fig. 4C). These results indicate that the inhibitory action of AM404 is not mediated through VR1 stimulation.

#### Experiment 5: the effects of AM404 are not mimicked by different classes of cannabinoid CB1 receptor agonists

As antagonism of the CB1 receptor did not indicate whether CB1 receptors were involved in the actions of AM404, we studied whether

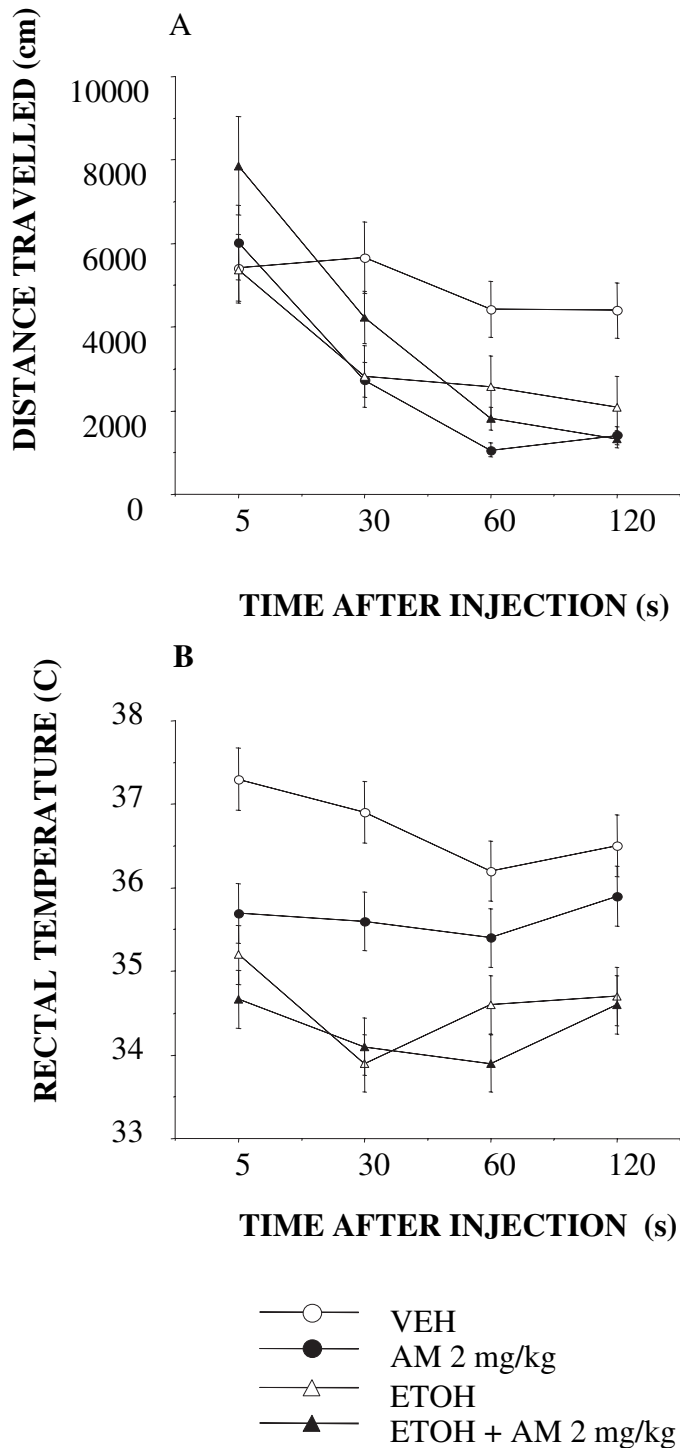


FIG. 2. Effects of acute administration of AM404 (2 mg/kg) 30 min prior to ethanol (ETOH) (2 g/kg) on locomotion and hypothermia. AM404 does not potentiate either ETOH sedation (A) or hypothermia (B). Data are means  $\pm$  SEM of 8–10 determinations/group. AM, AM404; VEH, vehicle.

cannabinoid CB1 receptor agonists mimicked the effects of AM404. Figure 5 shows the behavioral profile of the selective CB1 receptor agonist ACEA (Fig. 5A–C) and that of the non-selective CB1 receptor agonist WIN 55,212-2 (Fig. 5D–E). As shown in Fig. 5A, the administration of the selective CB1 agonist ACEA reduced ethanol self-administration at a dose of 2 mg/kg. As observed with AM404, ACEA did not modify operant responses for 0.2% saccharin (Fig. 5B).

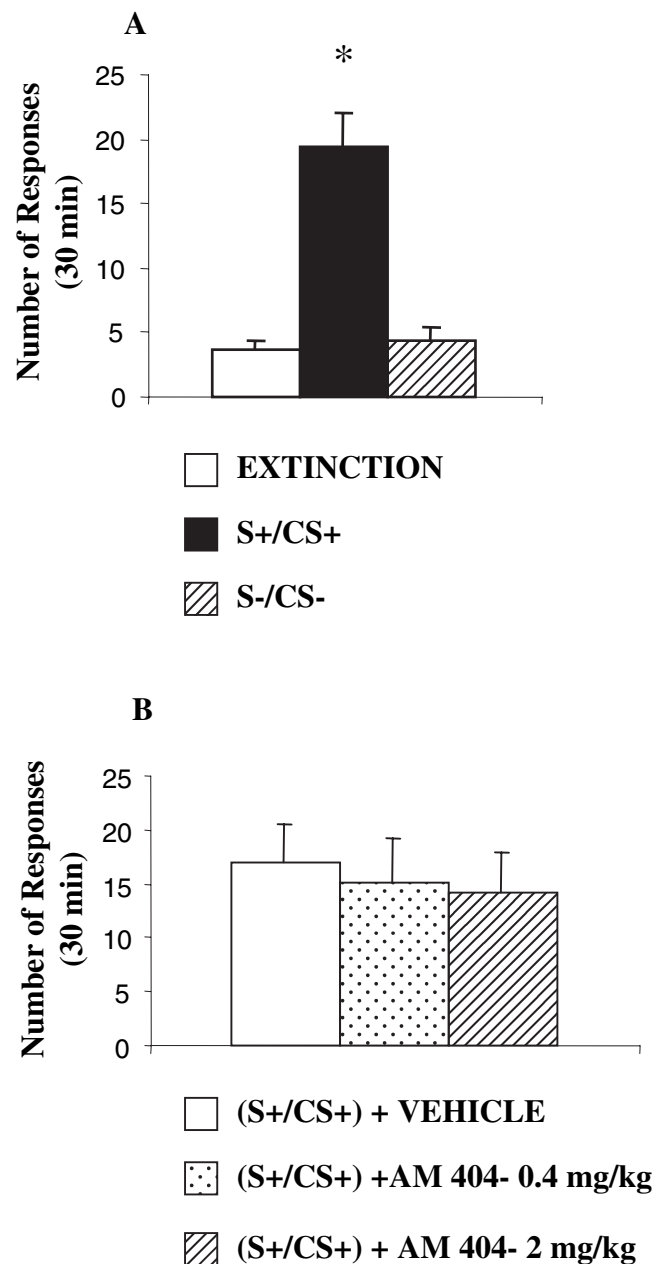


FIG. 3. Effects of acute administration of AM404 on cue-induced relapse to ethanol self-administration. (A) First test of cue-induced reinstatement of operant ethanol responding under conditions of S+/CS+ or S-/CS- stimuli. Responses to the active lever were significantly higher when compared with the last day of extinction. In contrast, under S-/CS- conditions, rats did not enhance operant responding when compared with the last day of extinction. (B) AM404 failed to alter the responses for ethanol-seeking behavior. Data are means  $\pm$  SEM of eight determinations/group. \* $P < 0.01$  vs. extinction. See Materials and methods for definitions.

All of the doses tested affected motor behavior in the open field test (Fig. 5C). However, the non-selective cannabinoid CB1 receptor agonist WIN 55,212-2 reduced both ethanol self-administration and operant responses for 0.2% saccharin (Fig. 5D and E, respectively). However, part of these effects might be masked by the motor depressant effects of this CB1 agonist at the doses effective on ethanol self-administration (Fig. 5F). A third class of cannabinoid receptor agonists, a classical cannabinoid HU-210, did not modify ethanol self-administration (Table 1) at doses that have been characterized as

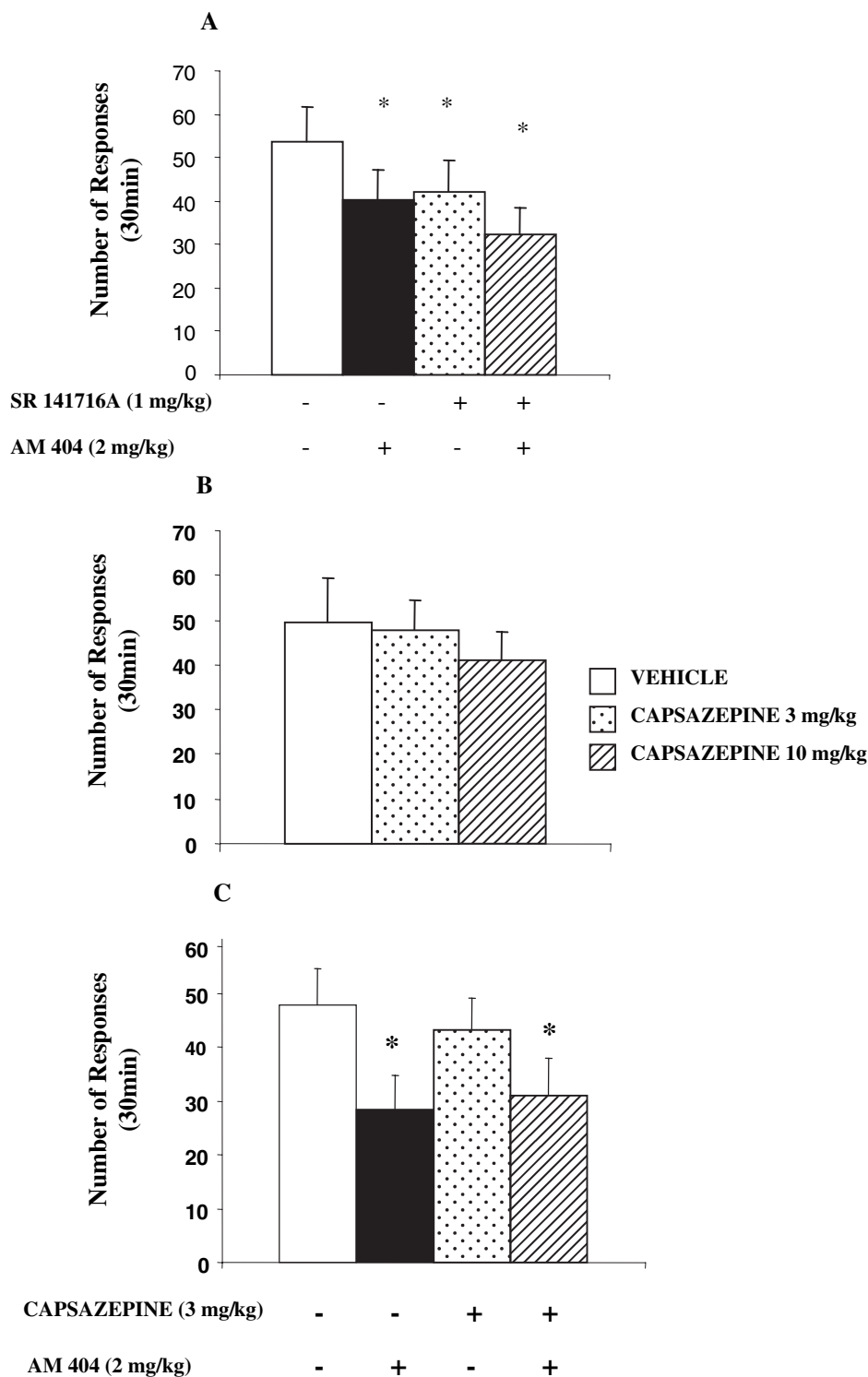


FIG. 4. (A) Effect of the pre-treatment with the selective CB1 receptor antagonist SR141716A on AM404-induced effects on ethanol self-administration. (B) Dose-response curve of the vanilloid VR1 receptor competitive antagonist capsazepine (3 and 10 mg/kg) on operant responding for ethanol. (C) The administration of capsazepine (3 mg/kg) does not prevent AM404 (2 mg/kg)-induced reduction on ethanol self-administration. Data are means  $\pm$  SEM of eight determinations/group. \* $P < 0.05$  vs. vehicle-treated animals.

devoid of side-effects on locomotion (Martin-Calderon *et al.*, 1998). Overall, these results indicate that the cannabinoid CB1 receptor is not the target of the action of AM404 as only the arachidonic acid derivative ACEA mimicked the actions of AM404.

## Discussion

The major finding of the present study is the demonstration that acute administration of the anandamide transport inhibitor AM404 reduces



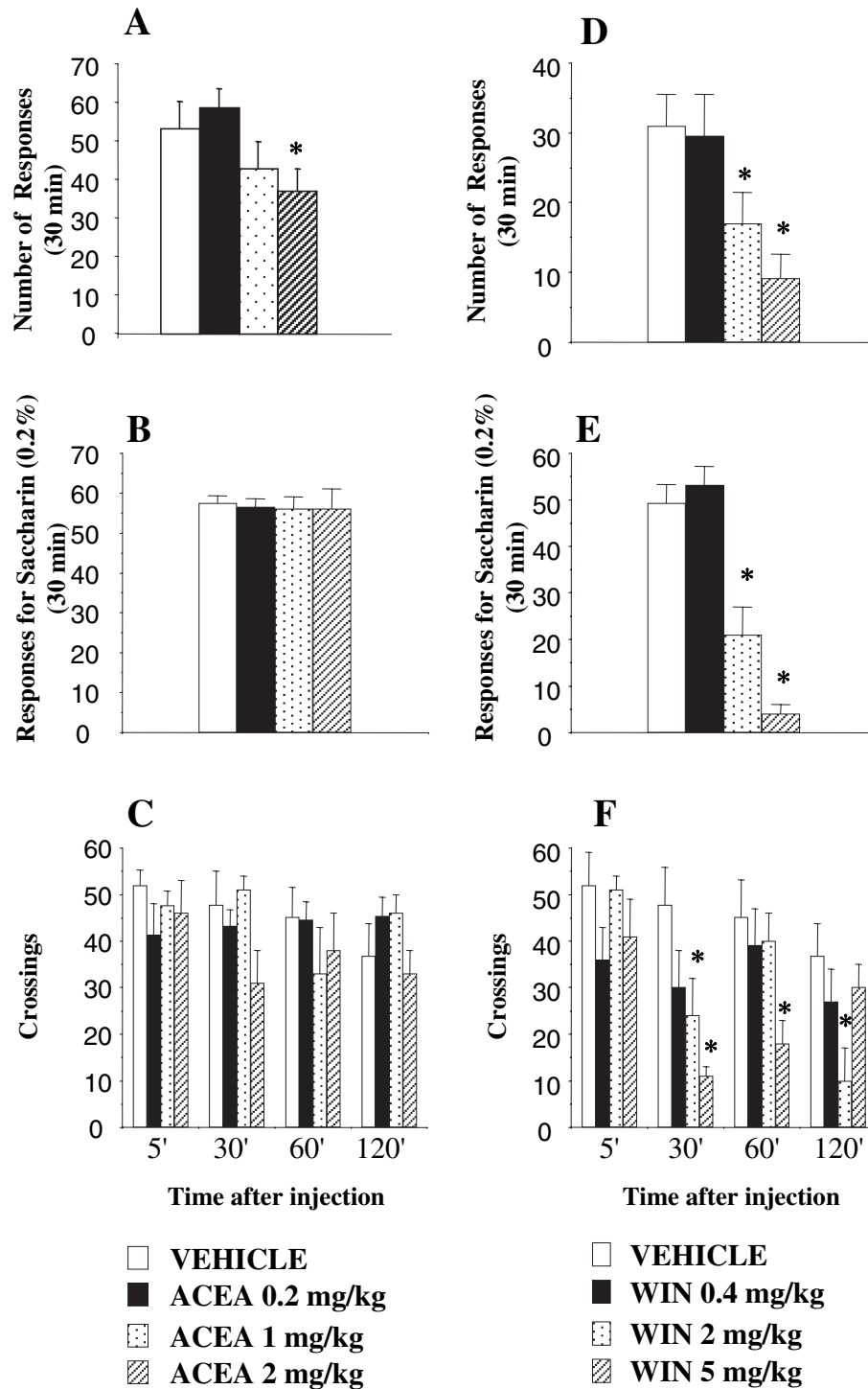


FIG. 5. Acute administration of the selective cannabinoid CB1 receptor agonist ACEA reduces ethanol self-administration (A) but not operant responses for saccharin (B) or locomotion in the open field (C). The potent non-selective cannabinoid CB1 receptor agonist WIN 55,212-2 (WIN) reduces both ethanol self-administration (D) and operant responses for saccharin (E). This agonist produces clear inhibition of locomotion at the doses tested (F). Data are means  $\pm$  SEM of 8–10 determinations/group. \* $P < 0.05$  vs. vehicle-treated animals.

ethanol self-administration under an operant conditioning schedule. This compound does not affect the relapse induced by contextual cues associated with ethanol. The effects of AM404 seem to be selective for ethanol, as it was unable to suppress responding for other reinforcers, such as saccharin or food intake, suggesting that this effect is not related to a decrease in a general motivational state. This is confirmed

by the lack of action of AM404 on the motivational properties of ethanol, as measured in the progressive ratio paradigm. This suppressive effect of AM404 on ethanol self-administration seems to be independent of the already known anandamide-induced motor impairment, as the lowest effective dose tested did not alter motor behavior in the open field. Moreover, the actions of AM404 were

TABLE 1. Acute administration of HU-210 30 min prior to the start of operant behavior sessions did not affect ethanol self-administration in Wistar rats

Test	Ethanol self-administration (responses per 30 min)
Vehicle	29.8 ± 6.4
HU-210 (4 µg/kg)	28.0 ± 5.6
HU-210 (10 µg/kg)	34.1 ± 5.9
HU-210 (20 µg/kg)	25.8 ± 5.3

Data are means ± SEM of eight animals tested in a within-subject Latin square design procedure.

found to be independent of a potentiation of the sedative effects of ethanol. Finally, neither experiments with cannabinoid CB1 receptor agonists nor with cannabinoid CB1 and CB2 receptor antagonists allowed us to obtain a direct pharmacological confirmation of the role of known cannabinoid receptors on the effects of AM404. The finding of a similar profile of effects using ACEA, a selective cannabinoid CB1 receptor ligand that shares the arachidonoyl moiety with both anandamide and AM404, suggests a common unknown target responsible for the effects of AM404 on ethanol self-administration. The lack of effects of WIN 55,212-2 and HU-210 at doses devoid of motor side-effects suggests that AM404 does not exert its actions through a CB1 receptor-mediated mechanism.

AM404 was the first synthetic inhibitor of anandamide uptake (Beltramo *et al.*, 1997) and it has been shown to potentiate many effects elicited by anandamide *in vitro* (Beltramo *et al.*, 1997) and *in vivo* (Beltramo *et al.*, 1997, 2000; Calignano *et al.*, 2000). As AM404 does not activate cannabinoid receptors (Beltramo *et al.*, 1997, 2000), the effects of this drug were suggested to result from the elevation of endogenous anandamide levels (Giuffrida *et al.*, 2000). However, recent findings suggest that AM404 also directly activates the vanilloid VR1 receptor (Zygmunt *et al.*, 1999, 2000; Smart *et al.*, 2000), complicating the identification of its mechanism of action on ethanol self-administration. However, the effect of AM404 was not reversed or enhanced by pre-treatment with the competitive vanilloid VR1 receptor antagonist capsazepine, indicating that the inhibitory action of AM404 is not mediated through VR1 stimulation and may be derived from other targets in the endocannabinoid system.

Following this rationale we studied the involvement of the cannabinoid CB1 receptor, the natural target of anandamide. In order to confirm its participation we first studied whether the cannabinoid receptor antagonist SR141716A reversed the actions of AM404. This pharmacological test was complicated by the inhibitory actions of SR141716A on ethanol self-administration that precluded the observation of a reversal of the actions of AM404. A second strategy was to compare the actions of AM404 with those of selective cannabinoid CB1 receptor agonists belonging to three of the four main classes of cannabinoid agonists: eicosanoids (ACEA), aminoalkylindoles (WIN 55,212-2) and classical cannabinoids (HU-210) (Rodriguez de Fonseca *et al.*, 2005). The effects of these compounds in ethanol self-administration are not similar to those of AM404. ACEA and WIN 55,212-2 reduced ethanol self-administration, although the component of motor inhibition of WIN 55,212-2 might be responsible for this effect. However, the classical cannabinoid receptor agonist HU-210 did not affect ethanol self-administration (Table 1). We replicated this finding in a separate study in Marchigian Sardinian alcohol-preferring rats (Cippitelli *et al.*, in preparation). These results indicate that the contribution of the CB1 receptors to AM404 cannot be supported.

The similar profile of actions observed after systemic administration of either cannabinoid CB1 receptor agonists or antagonist seems to be

challenging. It has been reported that both cannabinoid CB1 receptor agonists, such as tetrahydrocannabinol, CP55 940 and WIN 55,212-2, and cannabinoid receptor antagonist/inverse agonists, such as SR141716A, suppress operant behavior (Carriero *et al.*, 1998; Wiley *et al.*, 1995a,b; Mansbach *et al.*, 1996; Freedland *et al.*, 2000; Perio *et al.*, 2001; Jarbe *et al.*, 2003; Solinas *et al.*, 2003; De Vry & Jentsch, 2004; De Vry *et al.*, 2004; Cippitelli *et al.*, 2005). These reports stress the pleiotropic spectrum of actions found after the interference with endocannabinoid signaling. The complex roles of the endocannabinoid system on the regulation of GABA and glutamate synapses throughout the brain circuits processing the appetitive/motivational properties of ethanol might explain these findings (Rodriguez de Fonseca *et al.*, 2005). As an example, we have recently described that intracerebral injections of SR141716A only affect ethanol self-administration in rats when the CB1 antagonist is infused in the prefrontal cortex but not in the hippocampus or dorsal striatum (Hansson *et al.*, 2007). Moreover, in this study, local blockade of fatty acid amidohydrolase, the main enzyme that degrades anandamide, enhances ethanol self-administration when injected into the prefrontal cortex. However, we cannot exclude additional targets such as non-cloned cannabinoid-like receptors (Breivogel *et al.*, 2001; Hajos *et al.*, 2001) on which anandamide and WIN 55,212-2 may act. Thus, the present study stressed the need to clarify the growing complexity of endocannabinoid pharmacology, especially in the field of motivated behaviors.

Although the present results exclude VR1, CB1 and CB2 receptors as the targets of the effects of AM404, we cannot exclude the contribution of endocannabinoids elevated by AM404 to the present actions, especially because the endocannabinoid system has been recently implicated in the neuroadaptations that occur during acute alcohol exposure, alcohol dependence and abstinence. Several studies have documented that endocannabinoid transmission is acutely inhibited by ethanol (Ferrer *et al.*, 2007) and becomes hyperactive during chronic ethanol administration, as revealed by the increase in the levels of endocannabinoids and the down-regulation of CB1 receptors (Basavarajappa & Hungund, 2002). Thus, it is tempting to imagine that those compounds that increase endocannabinoid transmission, such as AM404, might be useful in reducing operant responses for ethanol. With the precautions derived from the non-CB1 profile of the effects of AM404, we propose that the increased levels of endogenous cannabinoids occurring during chronic ethanol administration contribute to facilitate the action of AM404; the neuroadaptations in the central nervous system associated with chronic ethanol intake lead to an increase in anandamide levels and this event could enhance the action of AM404 acting through the increased endogenous anandamide. However, we also demonstrate that the acute administration of AM404 was not able to suppress the relapse response for ethanol, i.e. the reinstatement of ethanol responding induced by the presentation of contextual cues associated with ethanol after a period of extinction. The differential response to AM404 in self-administration (presence of ethanol) and relapse (after a period of extinction conditions) conditions may have a neuropharmacological basis in the recently described changes in endocannabinoid levels after chronic ethanol exposure (Basavarajappa & Hungund, 2002; Gonzalez *et al.*, 2004). A possible explanation for these differences may reside in the probable alterations induced by chronically consumed ethanol in the functionality of the receptor systems mediating the central effects of ethanol that sustain ethanol-drinking behavior in rats. These neuroadaptation processes might result in a decreased potency and efficacy of the ligands. The increased levels of anandamide observed during ethanol consumption may return to basal levels or even disappear and thereby AM404 could not be acting in such a situation.

This hypothesis is supported by the results obtained recently by Gonzalez *et al.* (2004) who showed that the levels of endocannabinoids underwent significant changes in reward-related areas during relapse, showing the lowest values in this phase. The levels of both anandamide and 2-arachidonoyl-glycerol were significantly reduced when rats were allowed to relapse to alcohol consumption. Thus, the induction of compensatory mechanisms (such as up-regulation of the cannabinoid CB1 receptors and a decrease in the endocannabinoid levels) in the status of the endocannabinoid system may be determinant in the actions of AM404. The sensitivity to AM404 modulation of reward processes may be also dependent on the rate of motivation for the drug. The differential responses found after administration of AM404 in food and saccharin reinforcement with respect to ethanol might be explained by the different rewarding properties between natural rewards and drugs of abuse such as ethanol, the latter being more potent. In other words, AM404 may be efficacious only in those situations in which the motivation for the drug is stronger, indicating that this effect is linked to the strength of the hedonic properties of the reward rather than to a motivational state.

In conclusion, our results showing that AM404 administration reduced ethanol self-administration open a new line of research for the development of therapies to reduce ethanol intake in alcoholic non-abstinent patients.

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## Abbreviations

AM404, *N*-(4-hydroxyphenyl) arachidonoyl-ethanolamide; HU-210, 3-(1,1-dimethylheptyl)-(-)-11-hydroxy-delta 8-tetrahydrocannabinol.

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