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## The endogenous cannabinoid anandamide has effects on motivation and anxiety that are revealed by fatty acid amide hydrolase (FAAH) inhibition

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### Summary

Converging evidence suggests that the endocannabinoid system is an important constituent of neuronal substrates involved in brain reward processes and emotional responses to stress. Here, we evaluated motivational effects of intravenously administered anandamide, an endogenous ligand for cannabinoid-CB1 receptors, in Sprague-Dawley rats, using a place-conditioning procedure in which drugs abused by humans generally produce conditioned place preferences (reward). Anandamide (0.03 to 3mg/kg intravenous) produced neither conditioned place preferences nor aversions. However, when rats were pre-treated with the fatty-acid amide hydrolase (FAAH) inhibitor URB597 (cyclohexyl carbamic acid 3'-carbamoyl-3-yl ester; 0.3 mg/kg intraperitoneal), which blocks anandamide's metabolic degradation, anandamide produced dose-related conditioned place aversions. In contrast, URB597 alone showed no motivational effects. Like URB597 plus anandamide, the synthetic CB1-receptor ligand WIN 55,212-2 (50 to 300 µg/kg, intravenous) produced dose-related conditioned place aversions. When anxiety-related effects of anandamide and URB597 were evaluated in a light-dark box, both a low anandamide dose (0.3 mg/kg) and URB597 (0.1 and 0.3 mg/kg) produced anxiolytic effects when given alone, but produced anxiogenic effects when combined. A higher dose of anandamide (3 mg/kg) produced anxiogenic effects and depressed locomotor activity when given alone and these effects were potentiated after URB597 treatment.

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Finally, anxiogenic effects of anandamide plus URB597 and development of place aversions with URB597 plus anandamide were prevented by the CB1-receptor antagonist AM251 (3 mg/kg intraperitoneal). Thus, additive interactions between the effects of anandamide on brain reward processes and on anxiety may account for its aversive effects when intravenously administered during FAAH inhibition with URB597.

## Keywords

Endogenous cannabinoids; anandamide; FAAH; URB597; WIN 55,212-2; conditioned place preferences; anxiety; locomotor activity; rats

## Introduction

Converging evidence indicates that the endocannabinoid system is an important constituent of neuronal substrates involved in brain reward processes (Tanda et al., 1997, 2000; Ledent et al., 1999; Justinova et al., 2005b; Solinas et al., 2006) and emotional responses to stress (for review see: Valverde, 2005). However, little is known about direct effects of endogenous ligands for cannabinoid receptors on brain reward processes and emotional responses to stress.

Anandamide (N-arachidonylethanolamide) was the first identified endogenous ligand for brain cannabinoid receptors (Devane et al., 1992). It is produced upon demand through activity-dependent cleavage of membrane lipid precursors, and is released from cells immediately after its production. After release, anandamide is rapidly eliminated through a two-step process involving transport into cells followed by intracellular hydrolysis to arachidonic acid and ethanolamide, catalyzed by fatty-acid amide hydrolase (FAAH) (for review see: Piomelli, 2003). This rapid inactivation of anandamide has made study of its in-vivo behavioral and neurochemical effects difficult. However, selective inhibitors of FAAH, such as URB597 (cyclohexyl carbamic acid 3'-carbamoyl-3-yl ester) have recently been developed which can be used to magnify and prolong the effects of both endogenously released and exogenously administered anandamide (Kathuria et al., 2003; Lichtman et al., 2004; Mor et al., 2004; Fegley et al., 2005).

To date, there are only a few studies showing that endogenous cannabinoids such as anandamide interact and participate in the neuronal circuitry that signals rewarding events and controls emotional responses to stress. We recently demonstrated that intravenously-administered anandamide is an effective reinforcer of drug-taking behavior in squirrel monkeys (Justinova et al., 2005b) and produces THC-like discriminative effects in rats that are potentiated after FAAH inhibition by URB597 (Solinas et al., 2007). These effects of anandamide were blocked by the CB1-receptor antagonist rimonabant (SR141716). They were obtained with intravenous injection of anandamide, a route of administration that results in a more rapid onset and greater magnitude of effects than the more commonly used intraperitoneal route of administration (Smith et al., 1994). Intravenously-administered anandamide also produces a rapid, short-lasting increase in extracellular levels of dopamine (DA) in the nucleus accumbens shell of rats, which is magnified and prolonged by URB597 and blocked by rimonabant and is a neurobiological marker of most drugs abused by humans (Solinas et al., 2006).

In this study, we first investigated the effects of intravenously-administered anandamide with a place-conditioning paradigm, a procedure widely used in rodents to assess drug effects related to reward or aversion, that can be used to explore motivational brain reward processes related to drugs abused by humans (Mucha et al., 1982; Schechter and Calcagnetti, 1993; Tzschentke, 1998). Since anandamide is rapidly inactivated by FAAH enzymes, we also investigated the effects of intravenous anandamide after FAAH inhibition by URB597. For comparison, we

examined the motivational effects of intravenous administration of the synthetic CB1-receptor agonist WIN 55,212-2 with the same place-conditioning procedure. In addition, we attempted to reverse any effects obtained in the place-conditioning experiments with AM251, a selective CB1-receptor antagonist (an analog of rimonabant with greater selectivity for CB1 over CB2 receptors; Gatley et al., 1996, 1997).

It is known that the endocannabinoid system plays a modulatory role in emotional states such as anxiety and fear (Viveros et al., 2005). Several studies utilizing rodent models of anxiety or depression showed that FAAH inhibition produced anxiolytic-like effects (Kathuria et al., 2003; Patel and Hillard, 2006) and anti-depressant-like effects (Gobbi et al., 2005) in rats and mice, although these effects are not always found (Naidu et al., 2007). Also, the intra-dorsolateral periaqueductal injection of low dose of anandamide has been reported to produce anxiolytic-like effects when assessed with the elevated plus-maze (Moreira et al., 2007). Consequently, in separate groups of animals, we next investigated the effects of intravenously-administered anandamide alone and after FAAH inhibition by URB597 on anxiety-like responses at doses and combinations employed in the place-conditioning experiments. We used a light/dark test, a paradigm based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stressors, that is, novel environment and light (Crawley and Goodwin, 1980). Finally, to control for motor disturbances that anandamide or URB597 might induce, which could influence effects in the place-conditioning and light/dark box experiments, we investigated the effects of anandamide and URB597, alone and in combination, on spontaneous locomotor activity in an open-field test.

## Methods

### Animals

Subjects were Male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) weighing 300-325g at the beginning of the experiments. They were individually housed in a temperature- and humidity-controlled room on a 12-h light/dark cycle. Experiments were conducted during the light phase. Food and water were available ad libitum and each animal was adapted to daily handling for one week before the start of experiments. Animals used in this study were maintained in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and all experimentation was conducted in accordance with the guidelines of the Institutional Care and Use Committee of the Intramural Research Program, National Institute on Drug Abuse, NIH, and the Guide for Care and Use of Laboratory Animals (National Research Council 2003). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available.

### Surgical procedure

After acclimatization rats were anesthetized with an intraperitoneal (i.p.) injection of Equithesin (0.3 ml/100g) and under sterile conditions implanted with silastic catheters inserted into the right external jugular vein, as described previously (Solinas et al., 2003). Each rat was allowed to recover for approximately 1 week before the start of any experimental session.

### Drugs

Anandamide and AM251 were synthesized by Dr. Alexandros Makriyannis (University of Connecticut, Centre for Drug Discovery and Departments of Pharmaceutical Sciences and Molecular Cell Biology, Storrs, Connecticut USA and Northeastern University, Centre for Drug Discovery, Boston, MA, USA). WIN 55,121-22 was purchased from Sigma/RBI (St. Louis, MO, USA). URB597 was synthesized (Mor et al., 2004) at the Department of

Pharmacology, University of California, Irvine, CA, USA. Drugs were dissolved in a vehicle containing Tween 80 2%, ethanol 2%, and saline 96% and were injected in a volume of 1.0 ml/kg either i.v. or i.p., with the exception of URB597, which was dissolved in a vehicle containing 50% dimethylsulfoxide (DMSO) in saline and injected i.p.. Each experiment was done in a separate group of animals.

## Behavioral Procedures

### Place conditioning procedure

**Apparatus:** Locomotor activity boxes (Med Associates, St Albans, Vt., USA) were enclosed in sound-attenuation chambers (BRS/LVE, Laurel, Md., USA) with two boxes in each chest. A standard two-compartment place-preference insert (Med Associates) was situated inside each locomotor activity box. The two sides of the apparatus were differentiated by their floor type (mesh versus bar) and a small light was added on the wall of the sound-attenuation chambers on the side with the mesh floor, to increase the difference between the two sides. The illumination in the side with the mesh floor was approximately 1.3 lux. Each activity monitor consisted of a 16×16 infrared photocell detector array. These detectors measured the animal's position (left versus right side) and a computer tabulated time spent per side. Thus, time spent in one compartment could be deducted from the time spent in the other compartment (sum of both equaled 900 s). Before starting the experiment, each animal was randomly assigned to one of several different treatment groups (n= 9-11/group).

**Experimental procedure:** The general procedure consisted of three consecutive phases:

**-pre-test:** Rats were placed at the intersection of the two compartments with the guillotine door separating the two compartments raised and were allowed to explore both sides for 15 min. Time spent by the animal in each of the two compartments was recorded to monitor any initial preference for one side versus the other side. Animals showing a pronounced unconditioned preference for one compartment (more than 600 sec spent in one compartment) were excluded from the subsequent (conditioning) phase of the experiment.

**-conditioning:** Conditioning sessions were conducted over 3 consecutive days, with two sessions per day, using an 'unbiased' stimulus assignment procedure previously described (Le Foll et al., 2006). In the morning all rats received an i.v. injection of saline before being placed in one of the two compartments for 20 min, with the door separating the two compartments closed. Four hr later, the rats received an i.v. injection of anandamide (0.03 to 3mg/kg) or WIN 55,212-2 (15 to 300 µg/kg) or vehicle (Tween 80 2%, ethanol 2%, and saline 96%) and were placed in the opposite compartment for 20 min. URB597 (0.3mg/kg) was injected i.p., in the home cage, 40 min before vehicle or anandamide injection. For antagonism studies, AM251 (3mg/kg, i.p.) was co-administered with URB597 40 min before i.v. injection of vehicle or anandamide, or was administered alone 40 min before i.v. injection of vehicle or WIN 55,212-2. Drug-texture pairings were always counterbalanced.

**-test:** The day after the last conditioning session, a test session was conducted in which the animals did not receive any drug or vehicle treatment. During the test session, the door separating the two compartments was raised and the animals were placed at the intersection of the two compartments with access to both. Time spent by the animal in each of the compartments during a 15-min period was recorded.

### Light/dark test

**Apparatus:** Four clear acrylic shuttle boxes (MED-Associates, model ENV-010MC) were individually enclosed in sound-attenuation chambers in a darkened room illuminated only by

the screen of the computer. Shuttle boxes measured 40 × 16 × 21 cm and had an opaque black wall (2.5 cm thick) with a doorway (measuring 9 × 12 cm) between the compartments, such that the light compartment was 20 cm long and the dark compartment was 17.5 cm long. One compartment was lit by a bulb (100 mA, 28 V) covered by a white lens on the center of the wall at the far end of the compartment, 14.5 cm above the floor. The other compartment was unlit and had an opaque black covering around the outside of the box. The illumination (measured with a Sekonic light meter, model L308-B) was approximately 172 lux in the light side (in the centre) and approximately 5 lux in the dark side (facing the doorway). Each compartment had four photobeams spaced 5 cm apart, starting 5 cm from the center of the box.

**Experimental procedure:** On the day of the test, rats were transported to the darkened testing room and left in their home cages for 1 hr. Vehicle (Tween 80 2%, ethanol 2%, and saline 96%) or anandamide was administered i.v. immediately before testing. URB597 was injected i.p. in the home cage, 40 min before i.v. injection of vehicle or anandamide. Anxiety-like responses were recorded during three consecutive single daily sessions (DAYS 1-3). For antagonism studies, AM251 (3mg/kg i.p.) was co-administered with URB597 40 min before i.v. injection of anandamide or was administered alone 40 min before testing and anxiety-like responses were recorded during a single daily session. To start each session, a rat was placed in the light compartment, facing the corner of the wall with the light. During the 5-min test, total time spent (in sec.) and level of activity (in counts/min) in the light compartment was measured. Entry into a compartment, for purposes of measuring time spent in the compartment, was defined as the placement of all four paws in the compartment.

### Spontaneous motor activity in the open field

**Apparatus:** Locomotor-activity boxes (Med Associates, East Fairfield, VT, USA) were enclosed in sound-attenuation chambers, with two boxes in each chamber. A small light was added on the wall of the sound-attenuation chambers and the illumination was approximately 2.6 lux. The boxes (41 × 41 × 32 cm) were composed of clear acrylic and had sawdust bedding on the floor. Activity was measured with a 16 × 16 array of photobeams. Med Associates Open Field Activity Software recorded the distance traveled during the session.

**Experimental procedure:** Motor activity was measured in animals not habituated to the activity boxes during 60-min sessions. Locomotor activity was recorded during three consecutive single daily sessions (DAYS 1-3). At the beginning of each session, rats were placed in the centre of the testing box. Anandamide or vehicle (Tween 80 2%, ethanol 2%, and saline 96%) was administered i.v. immediately before placement of the animal in the activity box. URB597 was injected i.p. in the home cage, 40 min before vehicle or anandamide injection. Before starting the experiment, each animal was randomly assigned to one of different treatment groups (n= 6-7/group).

### Statistical analysis

All results are presented as group means (±SEM). The place conditioning data were analyzed by one-way analysis of variance (ANOVA) for repeated measures. Data from the light/dark test were analyzed by one-way analysis of variance (ANOVA). The locomotor-activity data were analyzed using repeated measures three-way analysis of variance (ANOVA), with URB597 pre-treatment, anandamide treatment and time as factors. Post-hoc comparisons, when appropriate, were performed by Newman-Keuls multiple comparisons test or by Tukey's multiple comparisons test. In all cases, differences with a P<0.05 were considered significant.

## Results

### Place conditioning study

No significant baseline differences were found between the time spent by the different groups of rats in the two compartments of the place-conditioning apparatus during the pre-conditioning session before conditioning sessions were started.

**Effects of anandamide on place conditioning**—Administration of different doses of anandamide (0.03 to 3 mg/kg i.v.) did not produce any significant change in time spent in the drug-paired compartment in the test session compared to the pre-test session [ $F_{9,81}=1.41$ ,  $P=ns$ , ANOVA] (Fig.1).

**Effects of anandamide on place conditioning when given after FAAH inhibition by URB597**—Significant treatment effects were found between subjects when time in the drug-paired compartment during the test and pre-test sessions was compared [ $F_{11,88}=14.61$ ,  $P<0.0001$ , ANOVA] (Fig.2). Post hoc analysis showed that when 0.3 or 3.0 mg/kg doses of anandamide were given during FAAH inhibition by URB597 (0.3 mg/kg), they produced significant decreases in time spent in the drug-paired compartment during the test session when compared with that in the pre-test session and with time spent by the vehicle group or URB plus vehicle group during the test session ( $P<0.05$  and  $P<0.001$ , respectively). Post hoc analysis also showed that when URB597 was administered before anandamide vehicle it did not affect time spent in the drug-paired compartment in the test session when compared with that in the pre-test session or with time spent by the vehicle group during the test session ( $P>0.05$ ) (Fig. 2)

**Effects of WIN55,212-2 on place conditioning**—As shown in Fig. 3, significant treatment effects were found between groups of rats when comparing the time spent in the drug-paired compartment before and after conditioning [ $F_{9,72}=16.01$ ,  $P<0.0001$ , ANOVA]. Post-hoc analysis showed that WIN55,212-2, at doses of 50, 150 and 300  $\mu\text{g}/\text{kg}$ , produced significant and dose-related decreases in time spent in the drug-paired compartment during the test session when compared with either time spent in that compartment in the pre-test session or with time spent in that compartment by the vehicle group during the test session ( $P<0.01$  and  $P<0.001$ , respectively).

**Effects of cannabinoid CB1-receptor blockade on the development of conditioned place aversions with anandamide after FAAH inhibition and with WIN 55,212-2**—When 3mg/kg of the CB1 antagonist AM251 was given to rats treated with either 0.3 mg/kg URB597 followed by 3 mg/kg anandamide or with 150  $\mu\text{g}/\text{kg}$  WIN 55,212-2, both of which produced significant conditioned place aversions, ANOVA for repeated measures showed significant treatment effects between groups of rats when comparing the time spent in the drug-paired compartment before and after conditioning sessions [ $F_{11,88}=4.510$ ,  $P<0.0001$  and  $F_{7,56}=11.72$ ,  $P<0.0001$ , respectively]. Post hoc analysis showed that AM251 prevented the development of conditioned place aversions induced by URB plus anandamide ( $P<0.05$ ) and by WIN55,212-2 ( $P<0.01$ ) (Figs. 4 and 5). Post hoc analysis also showed that AM251 administered before vehicle it did not affect time spent in the drug-paired compartment in the test session when compared with that in the pre-test session or with time spent by the vehicle group during the test session ( $P>0.05$ ) (Fig.4 and 5).

### Light/dark test

**Effects of URB597 on Anxiety-like behaviors**—On test day 1 and 2, one-way ANOVA (between subjects) revealed a significant effect of URB597 treatment on the time spent in the light compartment [ $F_{2,15}=7.52$   $P=0.0055$ ;  $F_{2,14}=5.485$   $P=0.0174$  respectively] (Fig. 6, upper

panel). Post hoc analysis showed that URB597 doses of 0.1 and 0.3 mg/kg significantly increased the time spent in the light compartment compared with vehicle treatment ( $P < 0.05$ ). On the third test day, the increase in time spent in the light compartment after 0.1 and 0.3 mg/kg URB597 was no longer significant. There was no significant effect of URB597 on level of activity during the three consecutive single daily test sessions (Fig. 6, lower panel).

**Effects of anandamide on Anxiety-like behaviors**—Fig. 7 shows the effects of anandamide, alone or in combination with URB597, in the light/dark test. On the first test day (DAY 1), one-way ANOVA (between subjects) revealed a significant effect of anandamide treatment on the time spent in the light compartment [ $F_{4,25} = 13.36$   $P < 0.0001$ ] as well as on the level of activity [ $F_{4,25} = 15.37$   $P < 0.0001$ ]. There was a significant increase in time spent ( $P < 0.05$ ) in the light compartment after administration of the lower dose of 0.3mg/kg anandamide compared with vehicle, but this anxiolytic-like effect no longer occurred after FAAH inhibition by URB597 (Fig. 7, upper panel). There was no significant effect of 0.3 mg/kg anandamide on level of activity (Fig. 7, lower panel). In contrast, administration of the higher 3mg/kg dose of anandamide did not increase time in the light compartment and after FAAH inhibition it produced the opposite response, i.e., a marked and significant decrease in the time spent ( $P < 0.001$ ) in the light compartment (an anxiogenic-like effect). The 3mg/kg dose of anandamide produced significant decreases in the level of activity ( $P < 0.001$ ) both alone and after FAAH inhibition.

On the second test (DAY 2), one-way ANOVA (between subjects) also revealed a significant effect of anandamide treatment on time spent in the light compartment [ $F_{4,22} = 8.923$   $P = 0.0002$ ], as well as on level of activity [ $F_{4,22} = 8.15$   $P = 0.0003$ ]. There was a significant increase in time spent ( $P < 0.01$ ) in the light compartment (an anxiolytic effect) after administration of the lower dose of 0.3mg/kg compared with vehicle, but, when this dose of anandamide was given after FAAH inhibition by URB597, there was no longer a significant effect compared to vehicle (Fig. 7, upper panel) and there was a significant decrease in time in the light compartment compared to 0.3 mg/kg anandamide alone ( $P < 0.01$ ). There was no significant effect of 0.3 mg/kg anandamide on the level of activity alone or after FAAH inhibition (Fig. 7, lower panel). Administration of the higher dose of 3mg/kg of anandamide, both alone and after FAAH inhibition by URB597, produced marked and significant decreases in time spent in the light compartment (anxiogenic effects) compared to vehicle ( $P < 0.001$ ). The 3mg/kg dose of anandamide also produced significant decreases in the level of activity ( $P < 0.001$ ), both alone and after FAAH inhibition, as it had on the first day.

By the third day (DAY 3), there was still a significant effect of treatment on the time spent in the light compartment [ $F_{4,22} = 5.34$   $P = 0.0037$ ] and on the level of activity [ $F_{4,22} = 7.9$   $P = 0.0004$ ]. Post hoc analysis showed a significant decrease in the level of activity at the higher 3mg/kg dose of anandamide, both alone and after FAAH inhibition with URB597, compared to vehicle ( $p < 0.01$ ).

**Effects of cannabinoid CB1-receptor blockade on the anxiogenic-like effect of anandamide after FAAH inhibition**—A 3mg/kg dose of the CB1 antagonist AM251 had no significant effect on time spent in the light compartment or in levels of activity (Fig. 8). As before (Fig.7), rats given 3 mg/kg anandamide after FAAH inhibition by 0.3 mg/kg URB597 showed significant decreases in time spent in the light compartment (anxiogenic-like effect) and decreases in levels of activity (Fig. 8). When rats given 3 mg/kg anandamide after FAAH inhibition by URB597 were pre-treated with 3mg/kg of AM251, ANOVA for repeated measures showed significant treatment effects between groups of rats on time spent in the light compartment [ $F_{3,18} = 3.78$   $P = 0.028$ ], as well as on level of activity [ $F_{3,18} = 8.7$   $P = 0.0010$ ]. Post hoc analysis showed that AM251 fully reversed both the anxiogenic-like effects and the



decreases in levels of activity that were produced by anandamide after FAAH inhibition with URB597 ( $P < 0.05$ ) (Fig.8)

### Locomotor activity in the open field

Fig. 9 shows the effects on locomotor activity of different doses of anandamide (AEA), given alone (upper panel) or in URB597 pre-exposed rats (lower panel) during three consecutive single daily sessions. On the first test day (DAY 1), three-way ANOVA showed a significant treatment-effect [ $F_{3,44} = 8.02$   $P = 0.00022$ ], a significant pre-treatment-effect [ $F_{1,44} = 15.4$   $P = 0.00029$ ], a significant time-effect [ $F_{5,220} = 221.6$   $P = 0$ ], a significant time  $\times$  treatment interaction [ $F_{15,220} = 14.3$   $P < 0.0001$ ] and a significant pre-treatment  $\times$  time interaction [ $F_{5,220} = 4.4$   $P = 0.0007$ ]. Post hoc analysis of data from the first 10 min after anandamide treatment showed a significant depression of locomotor activity in the 3mg/kg anandamide group and the URB597 plus 3mg/kg anandamide groups compared to the corresponding time-point for the other groups ( $P < 0.001$ ).

On the second test day (DAY 2), three-way ANOVA showed a significant pre-treatment-effect [ $F_{1,44} = 7.18$   $P = 0.01$ ], a significant time-effect [ $F_{5,220} = 143.1$   $P = 0$ ], a significant time  $\times$  treatment interaction [ $F_{15,220} = 3.3$   $P < 0.0001$ ] and a significant pre-treatment  $\times$  time interaction [ $F_{5,220} = 2.68$   $P = 0.02$ ]. Post hoc analysis of data from the first 10 min after anandamide treatment showed a significant depression of locomotor activity in the URB597 plus 3mg/kg anandamide group, but not in the 3mg/kg anandamide alone group, compared to the vehicle and the URB597 plus vehicle groups ( $P < 0.001$ ).

On the third test day (DAY 3), three-way ANOVA showed only a significant time-effect [ $F_{5,220} = 147.7$   $P = 0$ ] and a significant time  $\times$  treatment interaction [ $F_{15,220} = 2.46$   $P = 0.002$ ].

### Discussion

In the present study with Sprague-Dawley rats, the motivational effects of the endogenous cannabinoid receptor ligand anandamide in a place-conditioning procedure designed to measure rewarding or aversive effects of psychoactive drugs were compared with the emotional effects of anandamide in a light/dark box designed to measure anxiety. Intravenously administered anandamide, by itself, did not produce either conditioned place preferences or conditioned place aversions. However, when rats were pre-treated with the FAAH inhibitor URB597, which prevents intracellular inactivation of anandamide by FAAH and prolongs its behavioral and neurochemical effects (Katuria et al., 2003), anandamide produced dose-related conditioned place aversions. The synthetic CB1-receptor agonist WIN 55,212-2 also produced dose-related conditioned place aversions under the same experimental conditions. Doses of anandamide and the i.v. route of administration were chosen on the basis of our recent findings that i.v. anandamide produces THC-like discriminative-stimulus effects (Solinas et al., 2007) and produces dose-dependent increases in extracellular levels of dopamine in the nucleus accumbens shell (Solinas et al., 2006) in rats. Both effects are potentiated by FAAH inhibition with URB597. The aversive effects of anandamide during FAAH inhibition and of WIN 55,212-2 in the present study appeared to be mediated mainly by cannabinoid CB1 receptors, since pre-treatment with the cannabinoid CB1 receptor antagonist AM251 (Gatley et al, 1996; 1997) blocked the development of the conditioned place aversions induced by these drugs. Consistent with previous findings by Gobbi et al. (2005), URB597 by itself, at a dose (0.3 mg/kg) that produces almost complete inhibition of FAAH activity, did not produce either conditioned place preferences or conditioned place aversions.

In contrast to the present findings with anandamide during FAAH inhibition, no conditioned place aversions were found by Mallet and Beninger (1998) when they used an i.p., rather than an i.v., route of administration with anandamide in rats pre-treated with phenylmethylsulfonyl

fluoride, another potent, but not selective, FAAH inhibitor. Although procedural differences could be responsible for this discrepancy, it is more likely that, when injected i.p., anandamide availability is not sufficient to produce effects in the place preference procedure because of hepatic first-passage metabolism, which does not favor rapid entry of anandamide into the brain, together with likely higher peak blood concentrations of anandamide after i.v. injection. For example, we have recently demonstrated that high doses of anandamide can produce THC-like discriminative effects when injected i.v. but not when injected i.p. in rats (Solinas et al., 2007).

When administered i.v., anandamide shares several physiological and behavioral characteristics with other drugs of abuse. Intravenous anandamide activates dopaminergic transmission in the mesolimbic system, resulting in increased extracellular levels of dopamine in the shell of the nucleus accumbens of rats and this effect of anandamide is dramatically increased in magnitude and prolonged by FAAH inhibition with URB597 (Solinas et al., 2006). Also, i.v. anandamide is self-administered by a non-human primate, the squirrel monkey, with levels of drug-taking behavior higher than vehicle and comparable to those maintained by i.v. injections of either cocaine or  $\Delta^9$ -tetrahydrocannabinol (THC) under similar conditions (Justinova et al., 2005b). Further, after FAAH inhibition with URB597, anandamide produces dose-dependent THC-like discriminative-stimulus effects in rats trained to discriminate i.p. injections of THC from i.p. injections of vehicle (Solinas, et al., 2007). In spite of its abilities to increase extracellular dopamine levels in the shell of the nucleus accumbens and to produce THC-like discriminative effects in rats, i.v. anandamide did not induce conditioned place preferences in rats under our experimental conditions. It should be noted, that the synthetic CB1 receptor agonist WIN 55-212-2 also increases extracellular dopamine levels in the nucleus accumbens shell (Tanda et al., 1997) and produces THC-like discriminative effects in rats (Gobbi et al., 2005), but it did not induce conditioned place preferences in rodents in the present experiments or in previous experiments by others (Chaperon et al., 1998), except under restricted conditions such as enriched environments (Bortolato et al., 2006).

In contrast to mice, cannabinoid CB1 receptor agonists usually produce anxiogenic-like effects in rats (Haller et al., 2007). One possible explanation for the aversive effects of WIN 55,212-2 and of i.v. anandamide after FAAH inhibition in rats with our place-conditioning procedure could be anxiogenic effects that mask the development of positive place preferences. In our light/dark experiments, we found that i.v. anandamide given alone and after FAAH inhibition produced marked and significant anxiogenic-like effects at doses (3 mg/kg anandamide; 0.3 mg/kg URB597 plus 0.3 mg/kg anandamide; 0.3 mg/kg URB597 plus 3 mg/kg anandamide) that increase extracellular levels of dopamine in the shell of the nucleus accumbens (Solinas et al., 2006). Pre-treatment with a 3 mg/kg dose of AM251 significantly and completely reversed the anxiogenic effects of the high 3mg/kg dose of anandamide given after FAAH inhibition, showing that this effect was mediated by CB1 receptors.

The anxiogenic-like effects of the high 3mg/kg dose of anandamide, when given alone on the second and third day of testing or when given after FAAH inhibition on all three days of testing, were accompanied by decreases in the levels of activity in the light compartment of the light/dark box for three consecutive days. The 3 mg/kg dose of anandamide produced a similar decrease in locomotor activity in the open-field test, but only during the first day when it was given alone and during the first and second day when it was given after FAAH inhibition with URB597. The differences in the profile of effects in the two testing situations could be due to the different levels of activity in the two situations or to conditioned associations that develop when measuring activity in an anxiogenic environment.

It is possible that aversive effects of anandamide given alone and after FAAH inhibition may diminish or disappear after a priming injection or after chronic exposure. This view is supported by findings of Valjent and Maldonado (2000) that THC induced conditioned place preferences only in mice that received a priming injection of THC before being exposed to the drug-paired conditioning chamber, a procedure that may have reduced or avoided the possible aversive effects of the drug during conditioning. Whether reversal of place preference effects would occur in rats pre-exposed to anandamide plus URB597 combinations before exposure to the drug-paired conditioning chamber, is an interesting question that remains to be studied.

In contrast to the results obtained when URB597 was combined with i.v. anandamide, URB597 alone produced dose-dependent anxiolytic effects in the light/dark experiments, replicating previous findings by Kathuria et al. in rats (2003) and Patel and Hillard in mice (2006). Interestingly, a low dose of anandamide (0.3mg/kg, i.v.), by itself, produced an anxiolytic effect similar to that observed with 0.1 and 0.3 mg/kg URB597, supporting the hypothesis that endogenous elevations in brain anandamide levels are responsible for the anxiolytic effects of URB597. Importantly, when given alone, neither URB597 nor the low 0.3mg/kg dose of anandamide affected levels of activity in the light/dark experiments or locomotor activity in the open-field experiments, indicating selective anxiolytic-like effects. Moreover, when given alone, neither URB597 (0.3 mg/kg) nor the low 0.3 mg/kg dose of anandamide produced rimonabant-sensitive (CB1-receptor mediated) increases in dopamine levels in the nucleus accumbens shell (Solinas et al., 2006).

The effects of URB597 and anandamide in the light/dark test indicate a bell-shaped dose-response curve, with small increases in anandamide levels (URB597 alone or the low 0.3 mg/kg dose of anandamide alone) inducing anxiolysis, and large increases in anandamide levels (URB597 plus a larger 3mg/kg dose of anandamide) inducing anxiety. It is possible that URB597 affects anandamide signaling around presynaptic membranes of cells expressing both CB1 and GABA receptors (hence the tolerance), while exogenously administered anandamide produces more widespread increases in anandamide signaling around CB1 receptors located there and at all other CB1-receptor locations in the brain, including presynaptic membranes of cells expressing both CB1 and glutamate receptors (Haller et al., 2007), resulting in the different effects when URB597 and the low dose of anandamide were given alone and when URB597 was given in combination with different doses of intravenously-administered anandamide. Thus, URB597 alone may primarily increase anandamide availability at GABAergic synapses resulting in anxiolytic effects. Since GABAergic synapses are more sensitive to cannabinoid agonists than glutamatergic synapses, the low dose of anandamide may only have affected GABAergic synapses, again resulting in anxiolytic effects. However, when the low dose of anandamide was given after FAAH inhibition by URB597, availability of anandamide would increase and both GABAergic and glutamatergic synapses would be affected, resulting in the anxiolytic and anxiogenic effects counteracting each other. At the high dose of anandamide, CB1 receptors at glutamatergic synapses may be more strongly activated, resulting in the delayed appearance of anxiogenic effects on the second day of testing. Finally, when the high dose of anandamide was given after URB597, glutamatergic synapses may be fully activated and mild anxiolytic effects mediated by GABAergic synapses could be overwhelmed by strong anxiogenic effects mediated by glutamatergic synapses. Alternatively, the bell-shaped dose-response curve may be caused by interactions between the cannabinoid system and other systems, for example, vanilloid (Starowicz et al., 2007) or serotonergic (Braidia et al., 2007) systems.

In place-conditioning studies, cannabinoid CB1-receptor agonists such as THC seldom produce conditioned place preferences, except at low doses and under special conditions (Lepore et al., 1995; Valjent and Maldonado, 2000; Braidia et al., 2004; Le Foll et al., 2006), but higher doses of these drugs frequently produce dose-related conditioned place aversions

(present experiments and Parker and Gilles, 1995; Mc Gregor et al., 1996; Sañudo-Peña et al., 1997; Chaperon et al., 1998; Hutcheson et al., 1998). Environmental factors can influence responses in place-conditioning experiments with non-cannabinoid drugs such as amphetamine and kappa opioids (Bowling and Bardo, 1994; Smith et al., 2003) and environmental factors may also influence results with cannabinoids. For example, Bortolato et al. (2006) found that, in rats housed under enriched conditions, the putative anandamide uptake inhibitor AM404 (Beltramo et al., 1997) and the synthetic CB1-receptor agonist WIN55,212-2 both induced significant conditioned place preferences. Conversely, no place preferences or aversions were induced by AM404 or WIN55,212-2 in rats housed under normal non-enriched conditions. Thus, it is possible that in rats housed under enriched conditions, anandamide might induce conditioned place preferences rather than aversions in the presence of FAAH inhibition.

Concurrent exposure to other drugs may also influence responses in place-conditioning experiments with cannabinoids. For example, Le Foll and Goldberg (2006) reported a conditioned place preference at a low 0.1 mg/kg dose of THC and no conditioned preferences or aversions at higher 1 and 2 mg/kg THC doses in Sprague-Dawley rats. However, nicotine pre-exposed rats failed to develop conditioned place preferences with the low 0.1 mg/kg dose of THC and developed conditioned place aversions with higher 1 and 2 mg/kg doses of THC. These conditioned place aversions may reflect an increased sensitivity to cannabinoids after nicotine exposure.

Finally, significant differences have been reported between rat strains in the ability of cannabinoids to affect the threshold for intracranial self-stimulation (Lepore et al., 1996) and to support drug-taking and drug-seeking behavior (Deiana et al., 2006) and Sprague-Dawley rats have been reported to be insensitive to the positive reinforcing effects of WIN55,212-2 in intravenous self-administration experiments, unlike Long-Evans or Lister Hooded rats (Fattore et al., 2001; Deiana et al., 2006). However, Lecca et al. (2006) found positive reinforcing effects of WIN 55,212-2 in similar intravenous self-administration experiments in Sprague-Dawley rats.

In conclusion, activation of the endogenous cannabinoid system by i.v. anandamide has effects on motivation and anxiety that are revealed by fatty-acid amide hydrolase (FAAH) inhibition. Although intravenously administered anandamide and the FAAH inhibitor URB597, by themselves, had no motivational effects in the place-conditioning experiments, both URB597 and a low intravenous dose of anandamide had significant anxiolytic effects in the light/dark box experiments. When FAAH was inhibited by URB597, however, intravenously administered anandamide produced both dose-related anxiogenic effects and dose-related conditioned place aversions. Additive interactions between the effects of anandamide on motivational brain reward processes and on emotional responses to stress might account for its aversive effects when intravenously administered during FAAH inhibition with URB597.

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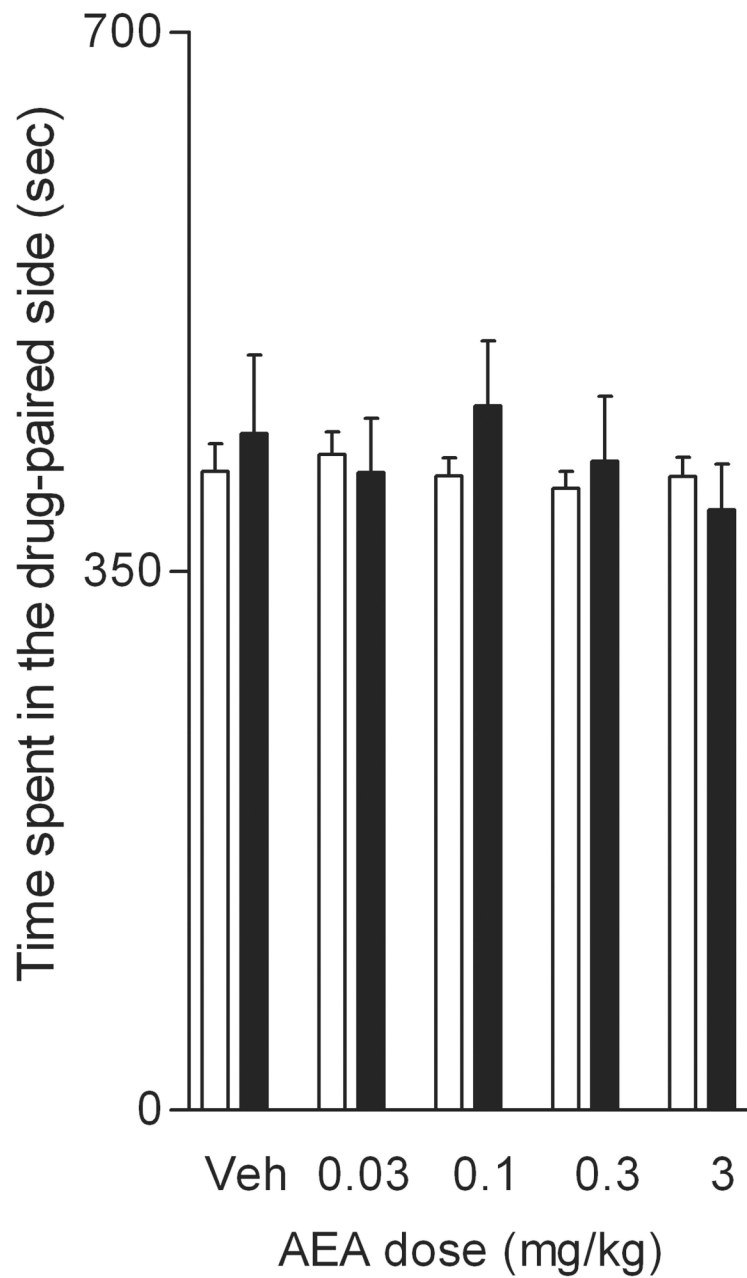
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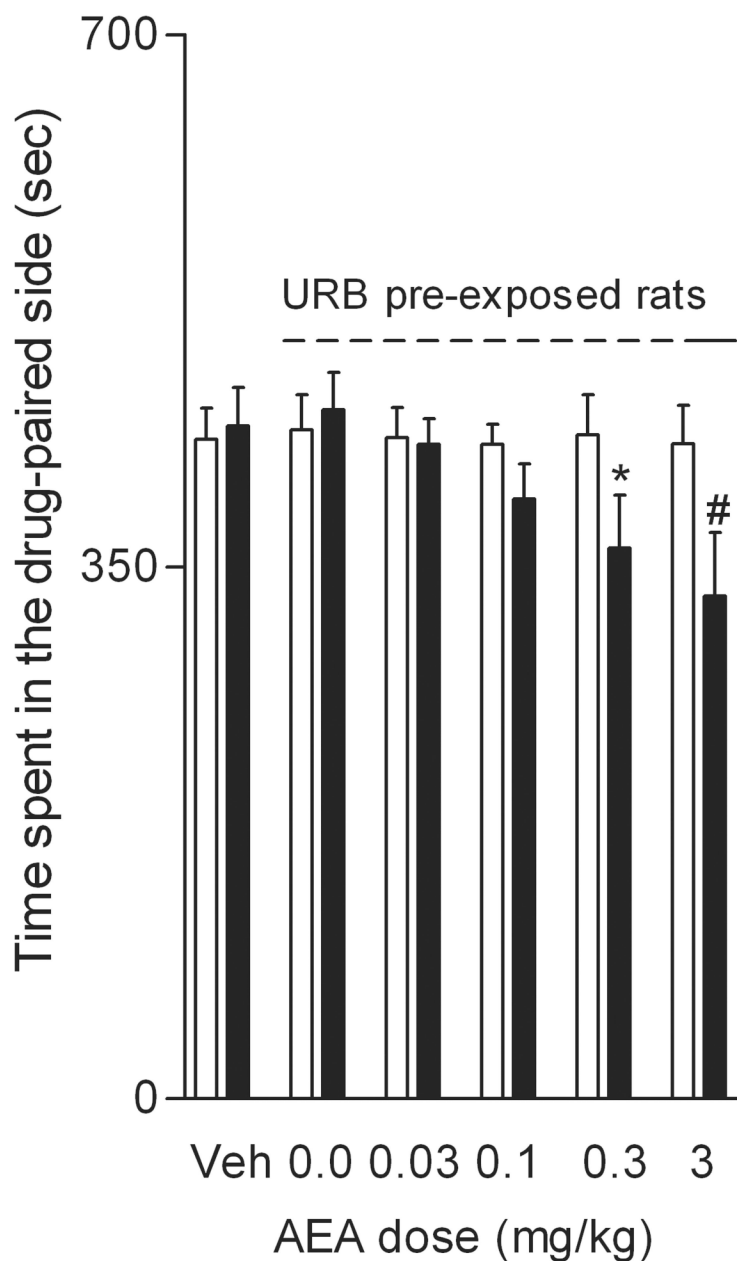
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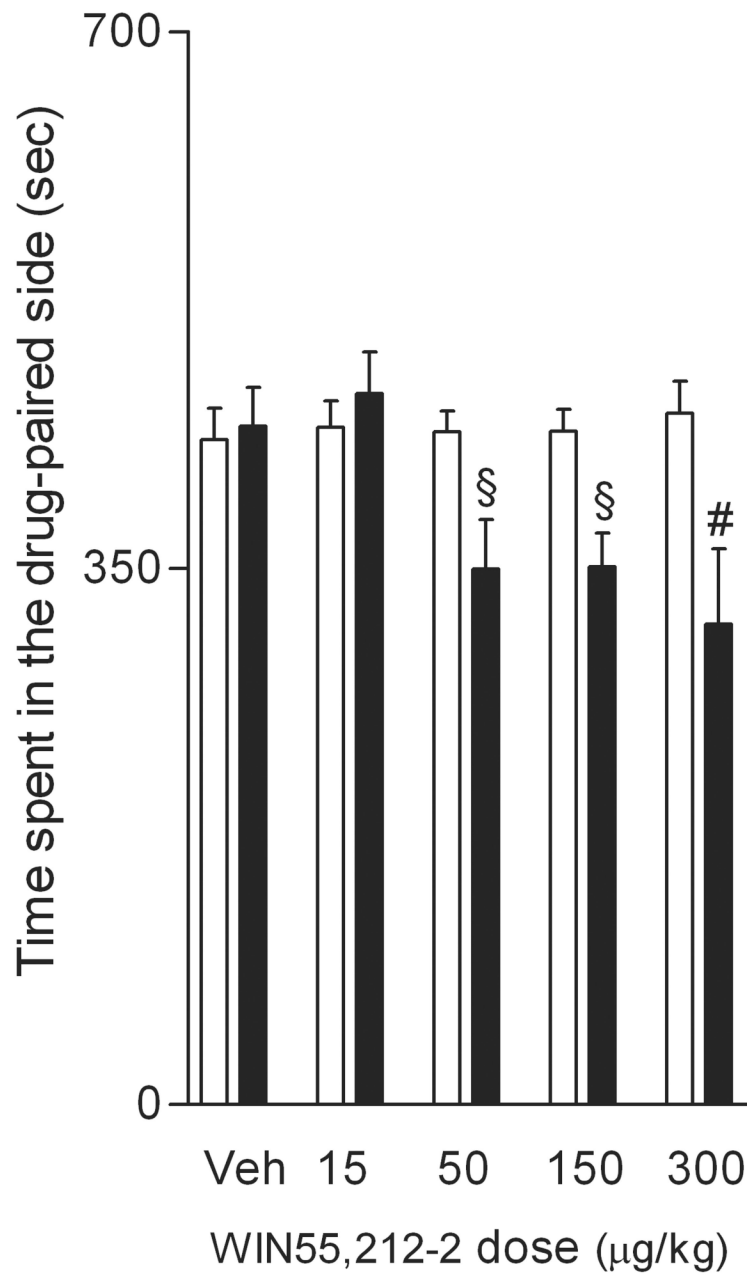


**Fig.1.** Effects of anandamide on time spent in sec (means  $\pm$  SEM n=9-11) in the drug-paired compartment during the 15-min pre-test session (open-bars) and the 15-min test session (closed-bars). Data were analyzed by one-way ANOVA for repeated measures between subjects. Anandamide (AEA) alone (0.03 to 3mg/kg) produced no significant conditioned place preferences or aversions (P=ns).

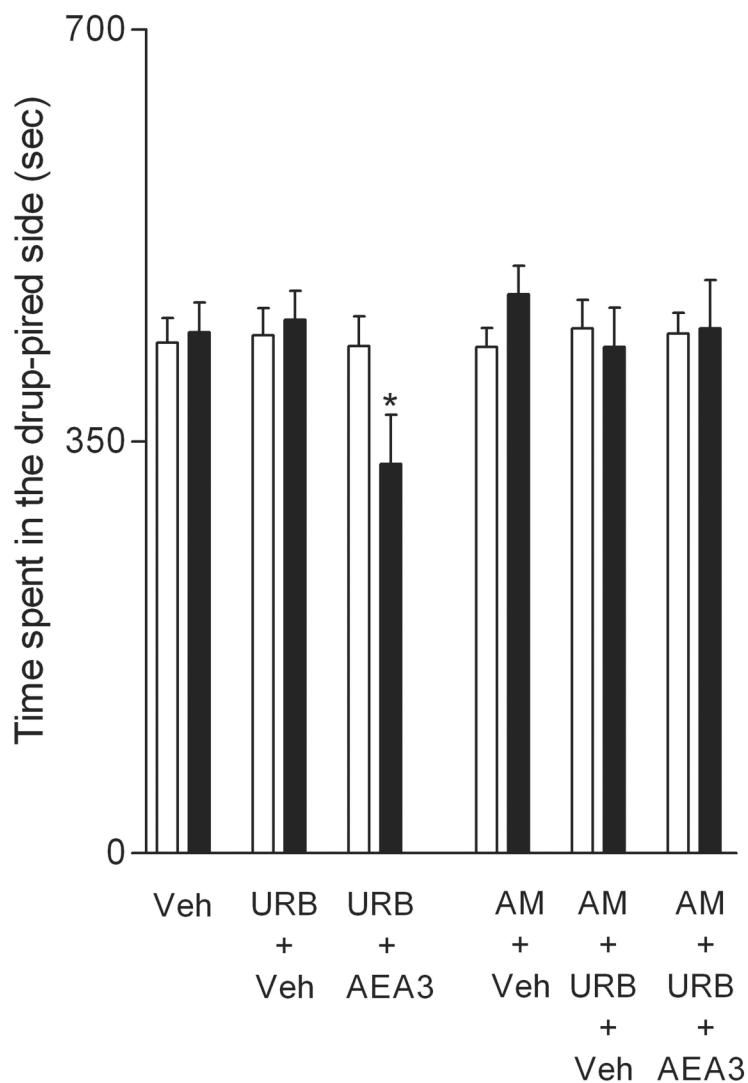




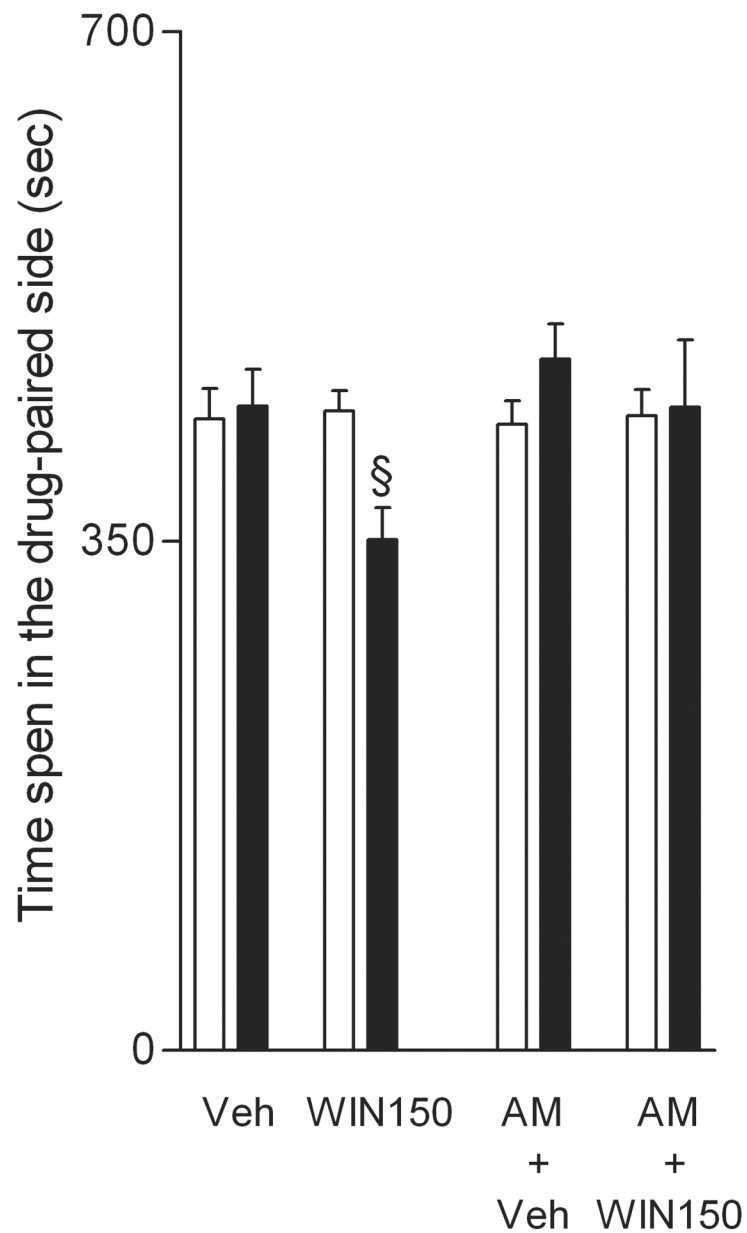
**Fig.2.** Effects of anandamide after FAAH inhibition by 0.3mg/kg URB597 on time spent in sec (means  $\pm$  SEM n=9-11) in the drug-paired compartment during the 15-min pre-test session (open-bars) and the 15-min test session (closed-bars). Data were analyzed by one-way ANOVA for repeated measures between subjects. Both the 0.3 and 3mg/kg doses of anandamide (AEA) induced significant conditioned place aversions. (\*,  $P < 0.05$  and #  $P < 0.001$  as compared with corresponding pre-test and with the vehicle or URB597 + vehicle test; Newman-Keuls multiple comparisons test).



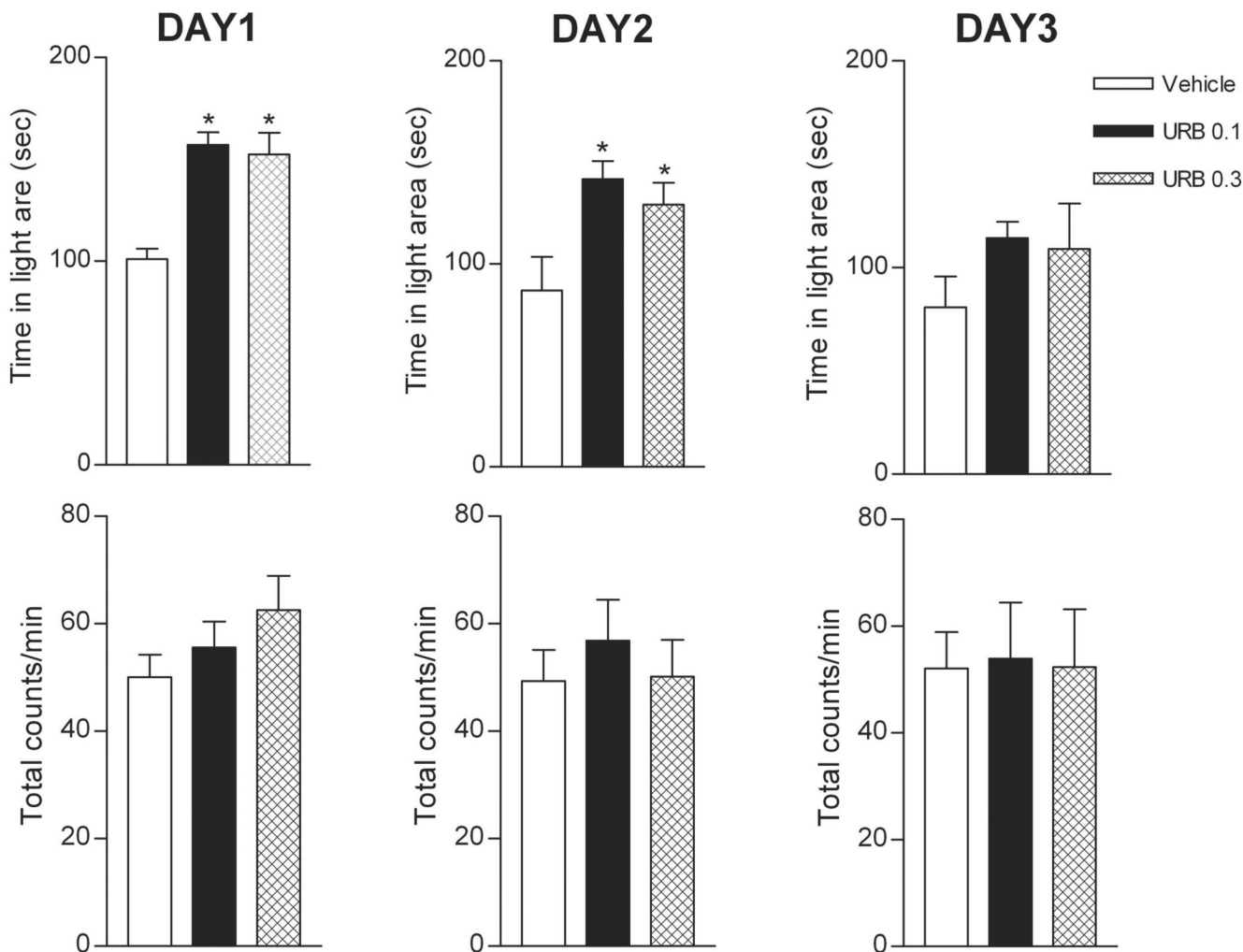
**Fig. 3.** Effects of WIN 55-212,2 on time spent in sec (means  $\pm$  SEM n=9-11) in the drug-paired compartment during the 15-min pretest session (open-bars) and the 15-min test session (closed-bars). Data were analyzed by one-way ANOVA for repeated measures. WIN55-212,2, at doses of 50, 150 and 300  $\mu\text{g}/\text{kg}$ , induced significant conditioned place aversions. (<sup>§</sup>  $P < 0.01$  and <sup>#</sup>  $P < 0.001$ , as compared with corresponding pre-test and with the vehicle test; Newman-Keuls multiple comparison test).



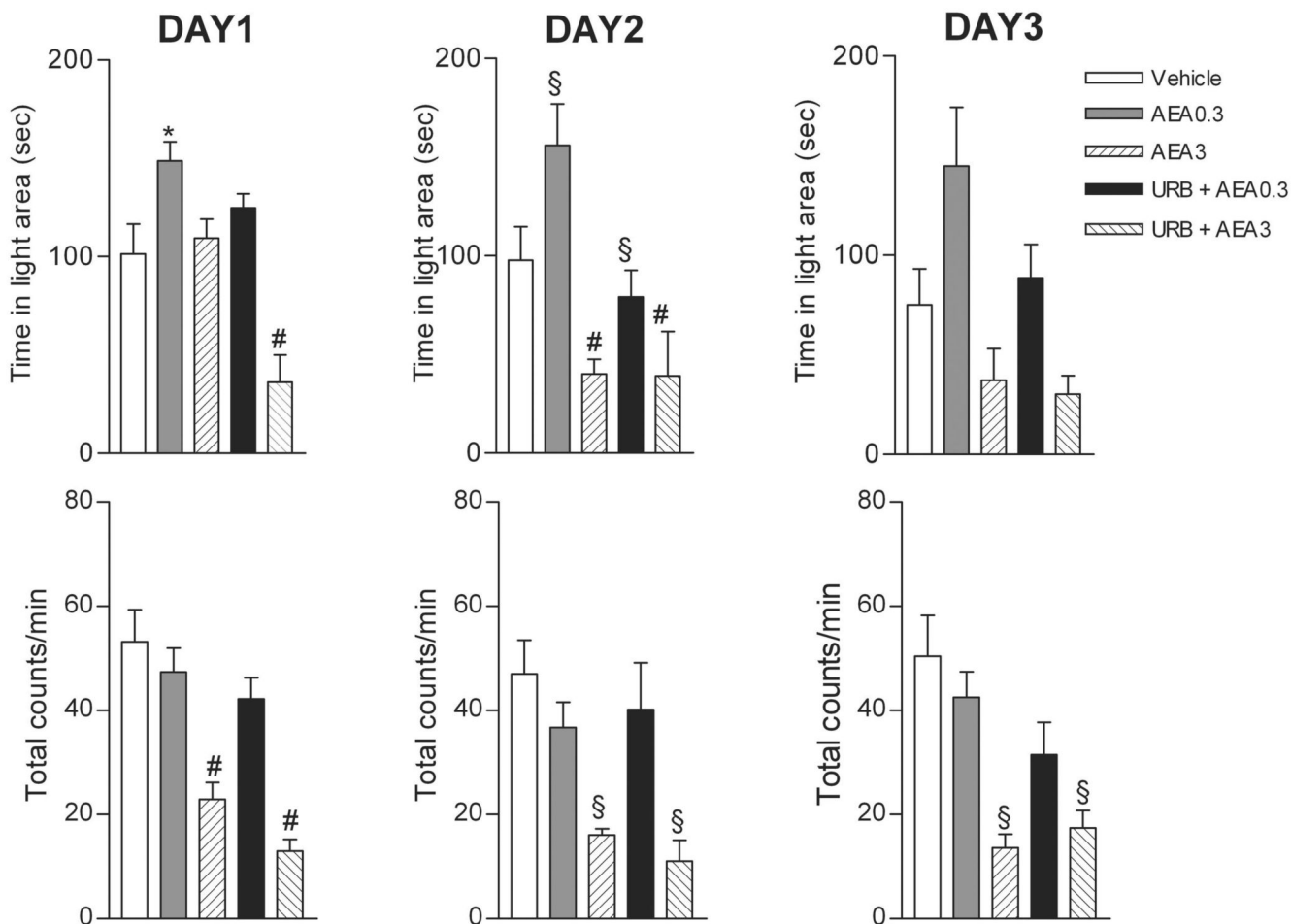
**Fig.4.** Effects of CB1 blockade on the development of conditioned place aversions induced by anandamide after FAAH inhibition by URB597. Time spent in sec (means  $\pm$  SEM  $n=9-11$ ) in the drug-paired compartment during the 15-min pre-test session (open-bars) and the 15-min test session (closed-bars) is shown. Data were analyzed by one-way ANOVA for repeated measures between subjects. 3 mg/kg of AM251 (AM) significantly blocked the development of the conditioned place aversions induced by 3 mg/kg of anandamide (AEA3) after FAAH inhibition by URB597 (URB). (\*,  $P<0.05$ , as compared with AM+URB+AEA3 test; Newman-Keuls multiple comparison test).



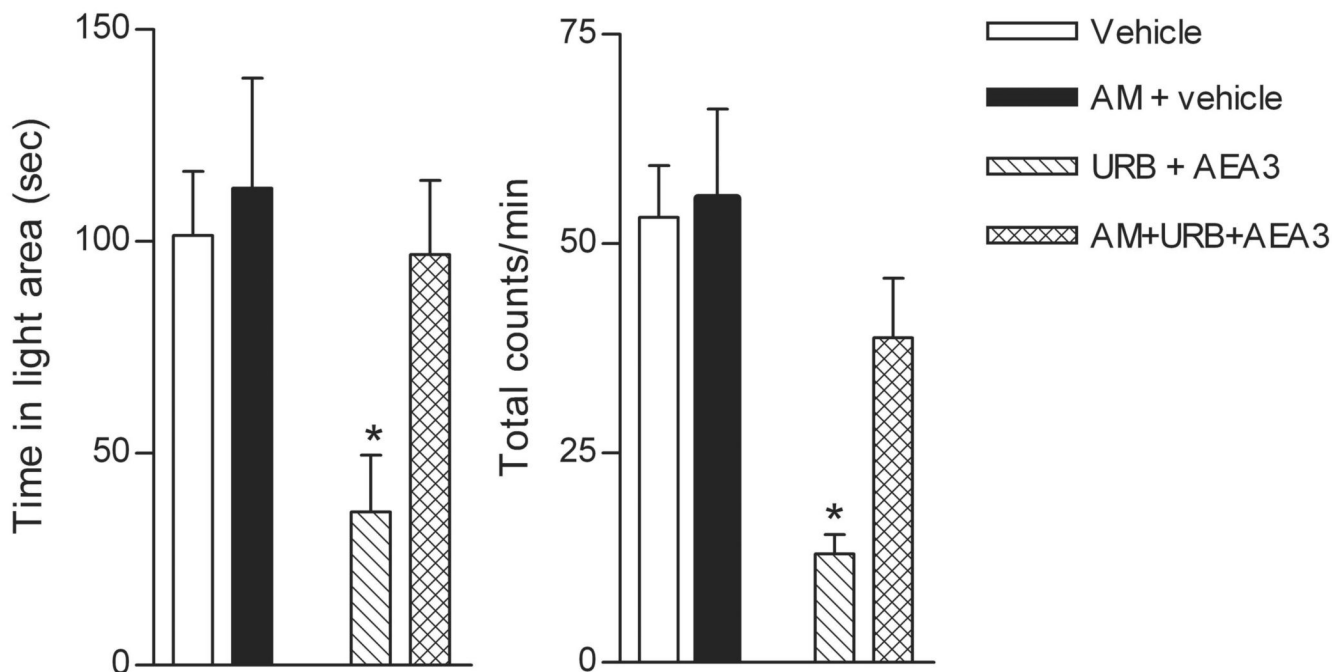
**Fig.5.** Effects of CB1 blockade on the development of conditioned place aversions induced by WIN 55,212-2. Time spent in sec (means  $\pm$  SEM n=9-11) in the drug-paired compartment during the 15-min pretest session (open-bars) and the 15-min test session (closed-bars) is shown. Data were analyzed by one-way ANOVA for repeated measures between subjects. 3 mg/kg of AM251 (AM) significantly blocked the development of conditioned place aversions induced by 150 $\mu$ g/kg of WIN55-212,2 (WIN150) (§,  $P < 0.01$ , as compared with AM3+WIN150 test, Newman-Keuls multiple comparisons test).



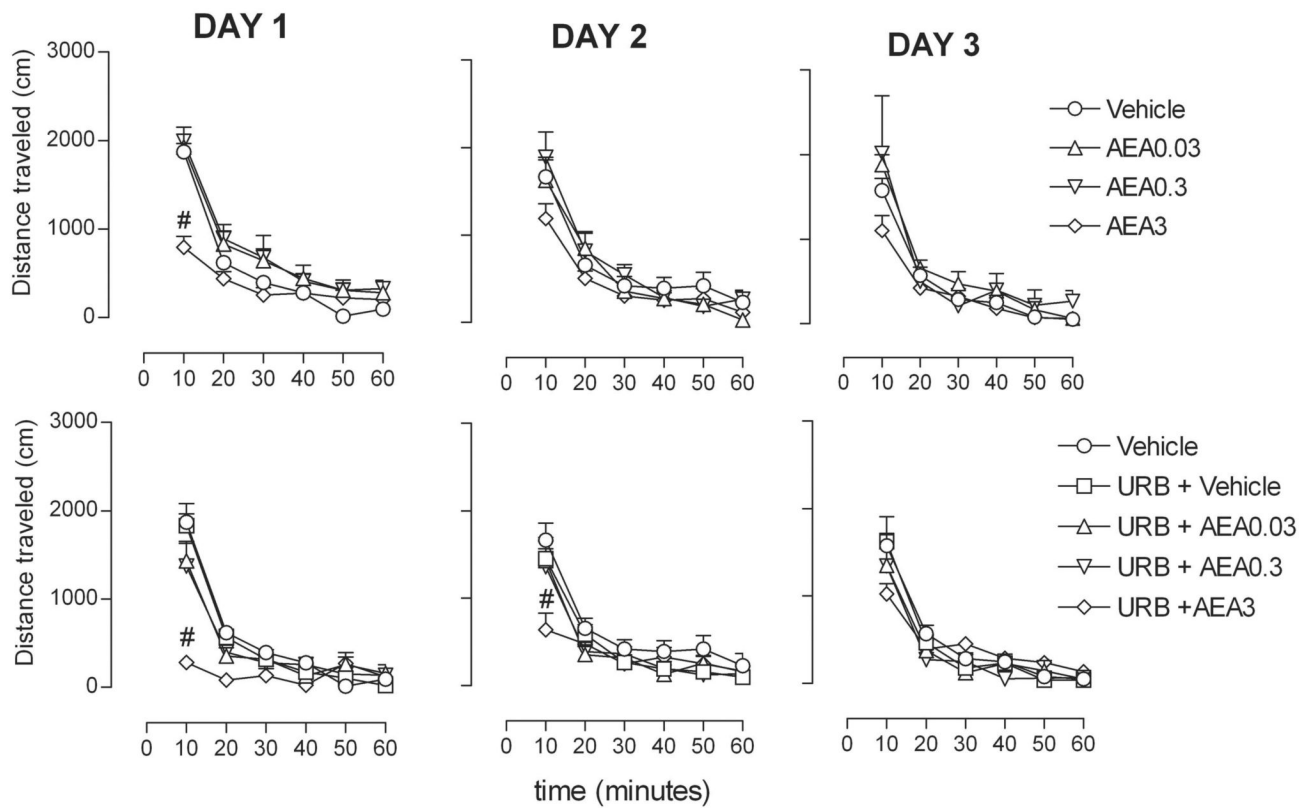
**Fig.6.** Effects of URB597 on anxiety-like behavior in the light/dark test. Rats received 0.1 or 0.3 mg/kg URB597 during three consecutive single daily sessions; (upper panel) time spent in the light compartment and (lower panel) level of activity (counts/min). Data represent the mean  $\pm$  SEM ( $n= 5-6$ ) and were analyzed by one-way ANOVA (\*  $P<0.05$  as compared with vehicle; Newman-Keuls multiple comparisons test).



**Fig.7.** Effects of anandamide (AEA), alone or in combination with URB597 0.3 mg/kg (URB), in the light/dark test. Rats received the drugs during three consecutive single daily sessions; (upper panel) time spent in the light compartment (sec) and (lower panel) level of activity (counts/min). Data represent the mean  $\pm$  SEM (n=5-6) and were analyzed by one-way ANOVA (\* P<0.05, § P<0.01, # P<0.001, as compared with vehicle and/or AEA0.3; Newman-Keuls multiple comparisons test;).

**Fig.8.**

Effects of CB1-receptor blockade on the anxiogenic-like and activity-decreasing effects produced by i.v. injection of 3 mg/kg anandamide in the light dark test after FAAH inhibition by URB597; (left panel) time spent in the light compartment (sec) and (right panel) level of activity (counts/min). Note that rats used for these CB1-receptor blockade experiments were experimentally naïve and these results represent a replication of findings with anandamide and URB597 shown in Fig. 7. Data shown represent the mean  $\pm$  SEM (n= 5-6) and were analyzed by one-way ANOVA. 3 mg/kg of AM251 (AM) significantly blocked the anxiogenic-like effect produced by 3 mg/kg of anandamide (AEA3) after FAAH inhibition by 0.3 mg/kg URB597 (URB). (\*  $P < 0.05$  as compared with AM+URB+AEA3; Newman-Keuls multiple comparisons test).



**Fig.9.**

Time course for effects of anandamide (AEA) alone (a) and after pre-treatment with URB597 0.3mg/kg i.p. (b) on locomotor activity. Mean ( $\pm$  SEM  $n=6-7$ ) distance traveled (cm) during each of three consecutive single daily sessions. Each session lasted 1h, and data are analyzed in 10-min blocks. Data were analyzed by three-way ANOVA for repeated measures. (#,  $P<0.001$  as compared to the corresponding time-point of the other groups; Tukey's comparisons test)