Neuropsychopharmacology (2006) 31, 2652–2659

www.neuropsychopharmacology.org

Anxiolytic-Like Properties of the Anandamide Transport Inhibitor AM404

Marco Bortolato[1,5], Patrizia Campolongo[2,5], Regina Anne Mangieri[1], Maria Luisa Scattoni[2], Roberto Frau[3], Viviana Trezza[2], Giovanna La Rana[4], Roberto Russo[4], Antonio Calignano[4], Gian Luigi Gessa[3], Vincenzo Cuomo[2] and Daniele Piomelli*[,1]

[1]Department of Psychiatry and Human Behavior, Center for Drug Discovery, University of California, Irvine, CA, USA; [2]Department of Human Physiology and Pharmacology, University of Rome 'La Sapienza', Rome, Italy; [3]Department of Neuroscience 'Bernard B. Brodie', University of Cagliari, Cagliari, Italy; [4]Department of Experimental Pharmacology, University of Naples, Naples, Italy

The endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) may contribute to the regulation of mood and emotion. In this study, we investigated the impact of the endocannabinoid transport inhibitor AM404 on three rat models of anxiety: elevated plus maze, defensive withdrawal and separation-induced ultrasonic vocalizations. AM404 (1–5 mg kg^{-1}, intraperitoneal (i.p.)) exerted dose-dependent anxiolytic-like effects in the three models. These behavioral effects were associated with increased levels of anandamide, but not 2-AG, in the prefrontal cortex and were prevented by the CB_1 cannabinoid antagonist rimonabant (SR141716A), suggesting that they were dependent on anandamide-mediated activation of CB_1 cannabinoid receptors. We also evaluated whether AM404 might influence motivation (in the conditioned place preference (CPP) test), sensory reactivity (acoustic startle reflex) and sensorimotor gating (prepulse inhibition (PPI) of the startle reflex). In the CPP test, AM404 (1.25–10 mg kg^{-1}, i.p.) elicited rewarding effects in rats housed under enriched conditions, but not in rats kept in standard cages. Moreover, AM404 did not alter reactivity to sensory stimuli or cause overt perceptual distortion, as suggested by its lack of effect on startle or PPI of startle. These results support a role of anandamide in the regulation of emotion and point to the anandamide transport system as a potential target for anxiolytic drugs.
Neuropsychopharmacology (2006) **31,** 2652–2659. doi:10.1038/sj.npp.1301061; published online 15 March 2006

Keywords: anandamide; anxiety; AM404; conditioned place preference; prepulse inhibition

INTRODUCTION

The endogenous cannabinoid anandamide is removed from the synaptic space by a high-affinity transport system present in neural and non-neural cells (Di Marzo *et al*, 1994; Beltramo *et al*, 1997; Hillard *et al*, 1997). Although the molecular identity of this putative transporter remains unknown, its functional properties have been partially characterized (for review, see Hillard and Jarrahian, 2003). These include substrate saturation at 37°C, stereoselective substrate recognition, independence from ion gradients, and pharmacological inhibition by agents such as *N*-(4-hydroxyphenyl)-arachidonamide (AM404), 5-biphenyl-4-ylmethyl-tetrazole-1-carboxylic acid dimethylamide (LY2183240), and *N*-(3-furylmethyl)-arachidonamide (UCM707) (Beltramo *et al*, 1997; Piomelli *et al*, 1999; de Lago *et al*, 2002; Moore *et al*, 2005). After internalization, anandamide is hydrolyzed by fatty-acid amide hydrolase (FAAH) (Désarnaud *et al*, 1995; Hillard *et al*, 1995; Ueda *et al*, 1995; Cravatt *et al*, 1996), an intracellular membrane-bound enzyme whose activity is selectively blocked by the compounds cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester (URB597) and 1-oxo-1-[5-(2-pyridyl)oxazol-2-yl]-7-phenylheptane (OL-135) (Kathuria *et al*, 2003; Lichtman *et al*, 2004) as well as by other less selective inhibitors (for review, see Piomelli, 2005).

Along with subtype-preferring cannabinoid ligands (Palmer *et al*, 2002) and mutant FAAH-deficient mice (Cravatt *et al*, 2001), blockade of anandamide deactivation has helped reveal potential functions of this lipid molecule in the regulation of dopamine signaling and long-term synaptic plasticity in the striatum (Giuffrida *et al*, 1999; Beltramo *et al*, 2000; Gerdeman *et al*, 2002; Azad *et al*, 2004) as well as acetylcholine signaling in the neocortex and hippocampus (Gifford *et al*, 1999; Tzavara *et al*, 2003; Steffens *et al*, 2003). Moreover, the hypoalgesic phenotype of FAAH-deficient mice (Cravatt *et al*, 2001) and the ability of FAAH inhibitors to alleviate pain, anxiety, and depression in rodent models (Kathuria *et al*, 2003; Lichtman *et al*,

*Correspondence: Dr D Piomelli, Department of Pharmacology, 3101 Gillespie NRF, University of California, Irvine, CA 92697-4625, USA, Tel: +1 949 824 9179, Fax: +1 949 824 6305,
E-mail: piomelli@uci.edu
[5]These authors contributed equally to this study.
Received 17 June 2005; revised 25 January 2006; accepted 26 January 2006
Online publication: 15 February 2006 at http://www.acnp.org/citations/Npp021506050395/default.pdf

2004; Hohmann *et al*, 2005; Gobbi *et al*, 2005) suggest that anandamide modulates the activity of neural circuits involved in the control of nociception, stress and emotion. These findings raise two clinically relevant questions. The first is whether inhibitors of endocannabinoid transport, which prolong anandamide's actions by preventing its access to intracellular FAAH (Beltramo *et al*, 1997; Kathuria *et al*, 2003), may exert anxiolytic-like effects. The second is whether such effects, if present, may be associated with other consequences of CB_1 receptor activation, such as perceptual distortion and liability to addiction (Hollister, 1998; D'Souza *et al*, 2005). To begin to address these issues, in the present study we have investigated the impact of a prototypical anandamide transport inhibitor, the compound AM404, on a series of behavioral models relevant to potential emotional and cognitive outcomes of endocannabinoid deactivation blockade.

MATERIALS AND METHODS

Animals

We used 10-day old Wistar pups for ultrasonic vocalization emission tests, adult male Sprague–Dawley rats (250–300 g) for startle and prepulse inhibition (PPI) tests, and adult male Wistar rats (200–350 g) for all other tests. Some of the rats tested for conditioned place preference (CPP) were housed under enriched conditions, consisting in exposing the rats for 6 weeks to a varied set of daily exchanged toys in a structured cage environment, as well as daily handling sessions. All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and those of the Italian Ministry of Health (D.L. 116/92).

Drugs

AM404 and WIN55,212-2 were from Tocris Cookson (Avonmouth, UK); morphine, diazepam, apomorphine and dizocilpine (MK801) from Sigma Aldrich (St Louis, USA); and rimonabant (SR141716A) from the National Institute on Drug Abuse (NIDA).

Endocannabinoid Analyses

At 45 min after injection with AM404 (2.5–10 mg kg^{-1}, intraperitoneal (i.p.)) or vehicle (polyethylene glycol, Tween 80, saline solution; 5:5:90 vol/vol), we anesthetized the rats with halothane and promptly decapitated them with a guillotine. Brains were removed within approximately 1 min after decapitation, frozen in 2-methylbutane and stored at -80°C until analysis. We placed frozen brains on a cutting block and sliced them into 1 mm sections. Prefrontal cortex was taken from approximately 2.20 to 4.20 mm rostral to bregma. Hippocampus was dissected from approximately 2.20 to 6.20 mm caudal to bregma. Thalamus was dissected from approximately 2.20 to 4.20 mm caudal to bregma. Tissues were immediately frozen on dry ice and stored at -80°C until analysis. We extracted endocannabinoids and related lipids with methanol–chloroform, fractionated them by open-bed silica gel chromatography and quantified them by isotope-dilution liquid chromatography/mass spectrometry (LC/MS), as described by Fegley *et al* (2004).

Elevated Plus Maze

Adult Wistar rats were placed in the central platform of the test apparatus (Pellow *et al*, 1985) and video recorded for 5 min in a dim light, sound-attenuated environment. The maze comprised two open arms (50×10 cm^2) and two closed arms ($50 \times 10 \times 40$ cm^3) that extended from a common central platform (10×10 cm^2). The apparatus, made of Plexiglas (gray floor, clear walls), was elevated to a height of 60 cm above the floor level. AM404 and vehicle were injected 30 min before testing. Behavioral analyses were performed by blinded observers, using the Observer 3.0 software (Noldus, Wageningen, the Netherlands). Percent time spent in open arms, number of head dips and stretched attend postures were measured as described by Griebel *et al* (2002).

Defensive Withdrawal

At 45 min after treatment, adult Wistar rats were placed in a small cylindrical stainless-steel chamber (11 cm diameter $\times$ 21 cm length) opened at one end, alongside one of the four walls of an open field (90×90 cm^2) (Takahashi *et al*, 2001), and video recorded for 15 min in a dim light, sound-attenuated environment. Behavioral analyses were performed by blinded observers, using the Observer 3.0 software (Noldus, Wageningen, the Netherlands). The latency to leave the chamber and the total amount of time spent in the open field were measured.

Isolation-Induced Ultrasonic Vocalizations

Vocalizations were recorded in 10-day-old Wistar rat pups, as described by Cagiano *et al* (1988). Briefly, a single male pup was randomly removed from each litter, weighed and placed in a shallow glass dish located 15 cm under a microphone, connected to a sound detector. Vocalizations were recorded 30 min after treatment, for 15 s and expressed as percent change from baseline.

Startle and PPI of Startle

The startle reflex was assessed as described by Bortolato *et al* (2005). At 45 min after treatment with either AM404 or vehicle, rats were placed in a startle reflex apparatus (Med Associates, St Albans, USA) for a 5 min acclimatization period with a 70 dB background noise, which continued for the remainder of the session. Each session consisted of three consecutive sequences of trials. During the first and the third sequence, the rats were presented with five pulse-alone trials of 115 dB. The second sequence consisted of 50 trials in pseudo-random order, including 12 pulse-alone trials, 30 trials of pulse preceded by 73, 76, or 82 dB prepulses (10 for each level of prepulse loudness), and eight no stimulus trials, where only the background noise was delivered. Intertrial intervals were selected randomly between 10 and 15 s. Acoustic devices and startle cages were connected to a computer, which detected and analyzed all chamber variables using customized software. Percent PPI was calculated with the following formula: 100−[(mean startle amplitude for prepulse–pulse trials/mean startle amplitude for pulse-alone trials) $\times$ 100].

CPP

CPP was evaluated as described by Gobbi *et al* (2005). The experiment consisted of three consecutive phases. The CPP apparatus consisted of four rectangular plastic shuttle boxes ($30 \times 60 \times 30\,cm^3$), each divided by a guillotine door into two distinct compartments of equal size, containing different visual and tactile cues. Visual cues were present on the walls, which were either brown or black and white striped; tactile cues were present in the floor, being either grid or chequered. All cues were present in the compartments in a counterbalanced order. The experimental room was sound attenuated and dimly lit. In phase I the animals were habituated to CPP boxes for 3 days and their initial side preferences were recorded. Phase II lasted 12 days and consisted of six alterned presentations of AM404 (1.25–10 mg kg^{-1}, i.p.), WIN55,212-2 (1 mg kg^{-1}, i.p.) or morphine (5 mg kg^{-1}, s.c.) and vehicle. Specifically, on odd days rats received one of the drugs or vehicle and were immediately placed in the nonpreferred compartment (separated from the other by a guillotine door) for 60 min, while on even days rats received vehicle and were placed in the preferred compartment for 60 min. On the test day (phase III), the animals were given no treatment and were placed in the cage with free access to both sides for 15 min. Drug-induced differences were assessed from the time spent in the nonpreferred compartment between postconditioning and preconditioning tests. Since environmental enrichment has been shown to positively affect mood and cognitive abilities in rats (Larsson *et al*, 2002; Renner and Rosenzweig, 1987), we conducted CPP tests in animals housed under both standard and enriched conditions.

Statistical Analyses

Results are expressed as the mean ± SEM of *n* experiments. All analyses were conducted using Statistica (Statsoft, Tulsa, USA). The significance of differences between groups was determined by one- or two-way analysis of variance (ANOVA) followed by Tukey's or Spjotvoll–Stoline's tests for multiple comparisons, as appropriate.

RESULTS

Effects of AM404 on Anandamide Levels in the Brain

The inhibitory effects of AM404 on anandamide transport have been previously characterized both *in vitro* and *in vivo* (Beltramo *et al*, 1997, 2000; Piomelli *et al*, 1999; Fegley *et al*, 2004). To assess its impact on brain anandamide levels, we administered AM404 to adult Wistar rats and measured endocannabinoid content in the prefrontal cortex, hippocampus and thalamus by LC/MS 45 min after injection. AM404 caused a dose-dependent increase in anandamide levels in prefrontal cortex ($F_{4,41} = 3.72$, $p < 0.05$; $p < 0.05$ for *post hoc* comparisons between vehicle and AM404 5 mg kg^{-1}), hippocampus ($F_{4,37} = 7.73$, $p < 0.001$; $p < 0.001$ for *post hoc* comparisons between vehicle and AM404 10 mg kg^{-1}) and thalamus ($F_{4,25} = 3.12$, $p < 0.05$; $p < 0.05$ for *post hoc* comparisons between vehicle and AM404 10 mg kg^{-1}) (Figure 1a–c). In contrast, the drug had no effect on the levels of 2-arachidonoyglycerol (2-AG), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) in any of the brain regions examined (Figure 1d–f and data not shown). As OEA and PEA are two FAAH substrates, which are markedly elevated after administration of FAAH inhibitors (Fegley *et al*, 2005), the present results confirm that AM404 does not affect FAAH activity *in vivo* (Fegley *et al*, 2004). As expected, the direct-acting cannabinoid agonist WIN55,212-2 (1 mg kg^{-1}, i.p.) had no effect on brain anandamide content (Figure 1a–c).

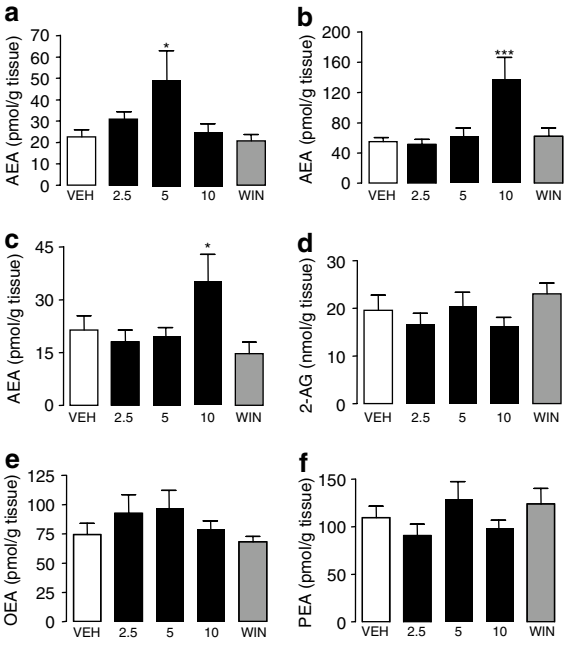

Figure 1 Levels of endocannabinoids and related compounds in select regions of the rat brain following administration of AM404 (2.5–10 mg kg^{-1}, i.p.) or WIN55,212-2 (WIN, 1 mg kg^{-1}, i.p.). Levels of anandamide (AEA) in (a) prefrontal cortex, (b) hippocampus, and (c) thalamus; levels of (d) 2-AG, (e) OEA, and (f) PEA in prefrontal cortex. Drugs were injected 45 min before killing. Results are expressed as mean ± SEM. Doses are in mg kg^{-1}. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to vehicle (VEH); $n = 6$–12 per group.

Effects of AM404 in the Elevated Plus Maze Test

Next, we examined whether the ability of AM404 to increase anandamide levels in the brain might be associated with a modulation of anxiety responses, as previously shown for the FAAH inhibitor URB597 (Kathuria *et al*, 2003). In a preliminary open field test, Wistar rats received different doses of AM404 (1–10 mg-kg^{-1}, i.p., 30 min before trials) to assess the effects of this drug on locomotor and exploratory activities. In agreement with previous results (Beltramo *et al*, 2000), only the dose of 10 mg kg^{-1} was found to elicit a significant reduction of locomotor activity (number of crossings: vehicle: 71.5 ± 7.9; AM404 2.5 mg kg^{-1}: 64.1 ± 9.3; AM404 5 mg kg^{-1}: 62.3 ± 11.2; AM404 10 mg kg^{-1}: 46.2 ± 7.8, $p < 0.05$ in comparison with vehicle; $n = 11$–16 per group; session duration: 10 min). We then tested the effects of AM404 in the elevated plus maze (Pellow *et al*, 1985). Rats treated with doses of AM404 that did not affect locomotor activity (0.5–5 mg kg^{-1}, i.p., 30 min before trials) spent a longer time in the open arm of the plus maze than did vehicle-injected rats (Figure 2a) ($F_{5,53} = 6.21$, $p < 0.001$;

$p<0.05$ for *post hoc* comparisons between vehicle and AM404 5 mg kg^{-1}). The clinically used anxiolytic diazepam produced a similar effect at the i.p. doses of 2.5 mg kg^{-1} (Figure 2a) and 5 mg kg^{-1} (data not shown). Moreover, AM404-treated animals exhibited a higher number of head dips (Figure 2b) ($F_{5,53}=5.86$, $p<0.001$; $p<0.01$ for *post hoc* comparisons between vehicle and AM404 5 mg kg^{-1}) along with a lower frequency of stretched attend postures (Figure 2c) ($F_{5,53}=9.52$, $p<0.001$; $p<0.01$ for *post hoc* comparisons between vehicle and AM404 5 mg kg^{-1}). Importantly, a nonanxiogenic dose of the CB_1 receptor antagonist rimonabant (1 mg kg^{-1}, i.p.) prevented all anxiolytic-like effects of AM404 (Figure 2d–f) (open arms time: $F_{3,36}=4.523$, $p<0.01$; head dips: $F_{3,36}=4.79$ $p<0.01$; stretched attend postures: $F_{3,36}=2.82$, $p<0.05$), indicating that such effects were mediated by CB_1 receptors.

Effects of AM404 on Defensive Withdrawal

As an additional test of the anxiolytic-like properties of AM404, we evaluated the effects of this agent on defensive withdrawal in Wistar rats (Takahashi *et al*, 2001). AM404 (1–5 mg kg^{-1}, i.p.) elicited a dose-dependent increase in time spent outside the box (Figure 3a) ($F_{2,27}=3.57$, $p<0.05$) and a dose-dependent decrease in latency to leave the box (Figure 3b) ($F_{2,27}=4.18$, $p<0.05$). Both responses were significantly reduced by rimonabant (1 mg kg^{-1}, i.p.) (Figure 3a and b) (total time: $F_{3,36}=3.69$, $p<0.05$; latency: $F_{3,36}=2.93$, $p<0.05$).

Effects of AM404 on Ultrasonic Vocalization Test

Concurrent results were obtained in the ultrasonic vocalization test in Wistar rat pups (Cagiano *et al*, 1988). In this test, AM404 (0.5–5 mg kg^{-1}, i.p.) exerted anxiolytic-like effects (Figure 3c) ($F_{5,60}=4.85$, $p<0.001$, $p<0.05$ for *post hoc* comparisons between vehicle and AM404 1 mg kg^{-1}; $p<0.01$ for *post-hoc* comparisons between vehicle and AM404 2 mg kg^{-1}) at doses that did not alter axillary temperature or locomotor activity (data not shown). These responses were comparable to those produced by diazepam (0.25 mg kg^{-1}, i.p.) ($p<0.01$; data not shown), and were abrogated by rimonabant (1 mg kg^{-1}, i.p.) (Figure 3c) ($F_{3,24}=6.54$, $p<0.001$). The inverse U-shaped effect of

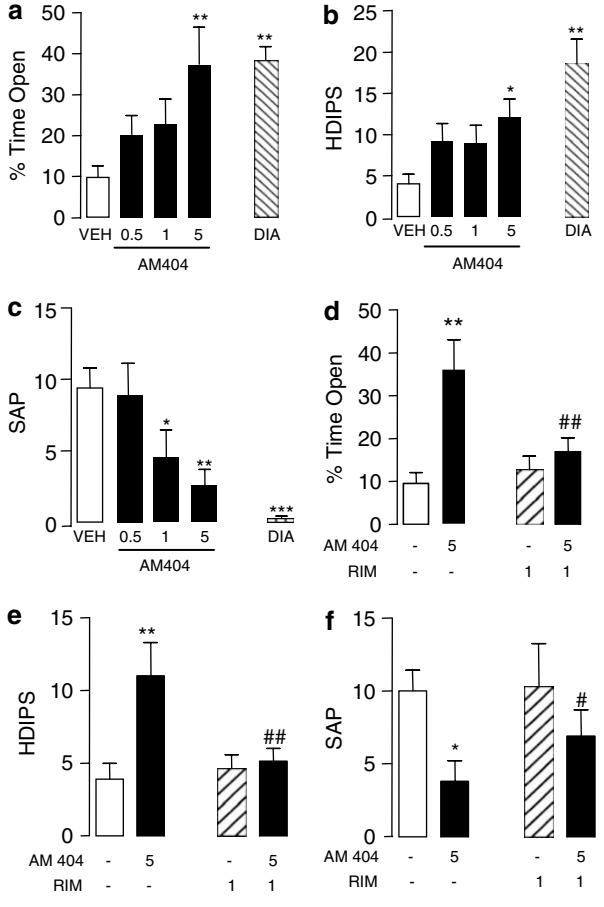

Figure 2 Effects of AM404 (0.5–5 mg kg^{-1}, i.p.) or diazepam (DIA, 2.5 mg kg^{-1}, i.p.) in the rat elevated plus maze test, and reversal of these effects by rimonabant (RIM). (a and d) AM404 and diazepam were injected 30 and 60 min before testing, respectively. Percent time spent in the open arms (%time open); (b and e) Number of head dips (HDIPS); (c and f) Number of stretched attend postures (SAP). $^{*}p<0.05$, $^{**}p<0.01$ compared to vehicle (VEH); $^{***}p<0.001$; $^{\#}p<0.05$; $^{\#\#}p<0.01$ compared to AM404 5 mg kg^{-1}; $n=8$–11 per group.

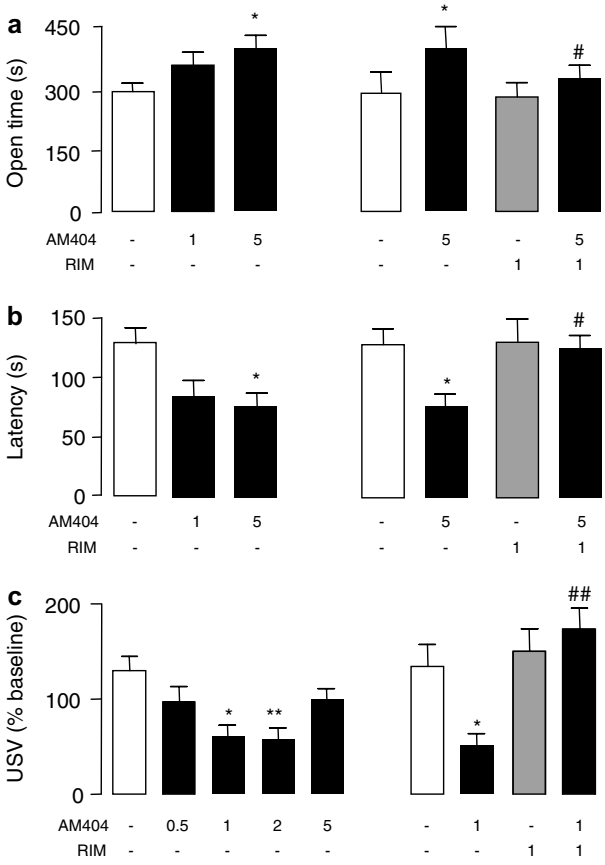

Figure 3 Effects of AM404 (1–5 mg kg^{-1}, i.p.) in the (a–b) rat defensive withdrawal and (c) ultrasonic vocalization tests, and reversal of these effects by rimonabant (RIM). (a) Time spent outside the box; (b) Latency to leave the box; (c) Ultrasonic vocalizations in 10-day old rat pups. $^{*}p<0.05$, $^{**}p<0.01$ compared to vehicle (VEH); $^{\#}p<0.05$; $^{\#\#}p<0.01$ compared to AM404 (5 mg kg^{-1} in defensive withdrawal and 1 mg kg^{-1} in ultrasonic vocalizations); $n=10$–12 per group.

AM404 observed in this test was likely due to a greater sensitivity of the pups to the effects of the drug.

Effects of AM404 on Startle and PPI of Startle

To determine whether the anxiolytic-like actions of AM404 might be associated with altered vigilance and reactivity to sensory stimuli, a common effect of both anxiolytic drugs and cannabinoid agonists (Koelega, 1989; Bahri and Amir, 1994), we measured startle magnitude in Sprague–Dawley rats after administration of AM404 (2.5–10 mg kg^{-1}, i.p.), WIN55,212-2 (5 mg kg^{-1}, i.p.) or diazepam (2.5–5 mg kg^{-1}, i.p.). Diazepam increased startle latency at the 2.5 and 5 mg kg^{-1} doses (data not shown), and decreased startle amplitude at the dose of 5 mg kg^{-1} (Figure 4a). WIN55,212-2 produced a significant reduction of startle amplitude, but did not affect startle latency (Figure 4a and data not shown) ($F_{5,62} = 4.2$, $p < 0.01$, $p < 0.05$ for *post hoc* comparisons between diazepam and vehicle and WIN55,212-2 and vehicle) (Bortolato *et al*, 2005; Abduljawad *et al*, 1997). By contrast, AM404 did not affect any startle-related parameter at the doses tested (Figure 4a). We then examined the impact of AM404 (2.5–10 mg kg^{-1}, i.p.) on PPI of the startle reflex, the disruption of which is widely utilized as a model of perceptual distortion (Swerdlow *et al*, 2000). We compared the effects of AM404 with those of WIN55,212-2 (1 mg kg^{-1}, i.p.), apomorphine (0.25 mg kg^{-1}, s.c.) and dizocilpine (0.1 mg kg^{-1}, s.c.). AM404 was ineffective in this test at all doses examined (Figure 4b). As previously shown, WIN55,212-2 was also unable to disrupt PPI (Bortolato *et al*, 2005), while dizocilpine significantly decreased it (Figure 4b) ($F_{2,38} = 5.26$; $p < 0.01$); a similar decrease was observed with apomorphine ($F_{2,38} = 3.77$; $p < 0.05$) (data not shown).

Effects of AM404 on CPP

Finally, we examined the motivational effects of AM404 in the CPP test, and compared them to those produced by WIN55,212-2 (1 mg kg^{-1}, i.p.) and morphine (5 mg kg^{-1}, s.c.). As shown in Figure 5a, Wistar rats housed under standard nonenriched conditions exhibited a significant shift in preference towards the morphine-associated compartments ($F_{6,48} = 6.89$, $p < 0.001$ for main treatment effect; $p < 0.05$ for *post hoc* comparison between vehicle and morphine), but not to the compartments associated with either AM404 (1.25–10 mg kg^{-1}, i.p.) or WIN55,212-2. In contrast, rats housed under enriched conditions displayed a significant shift in preference toward the environment associated with AM404, WIN55,212-2, or morphine (Figure 5b). In particular, enriched AM404-treated rats (1.25–10 mg kg^{-1}, i.p.) exhibited a dose-dependent shift in preference, which was significantly higher than controls for the 2.5 mg kg^{-1} dose (Figure 5b)

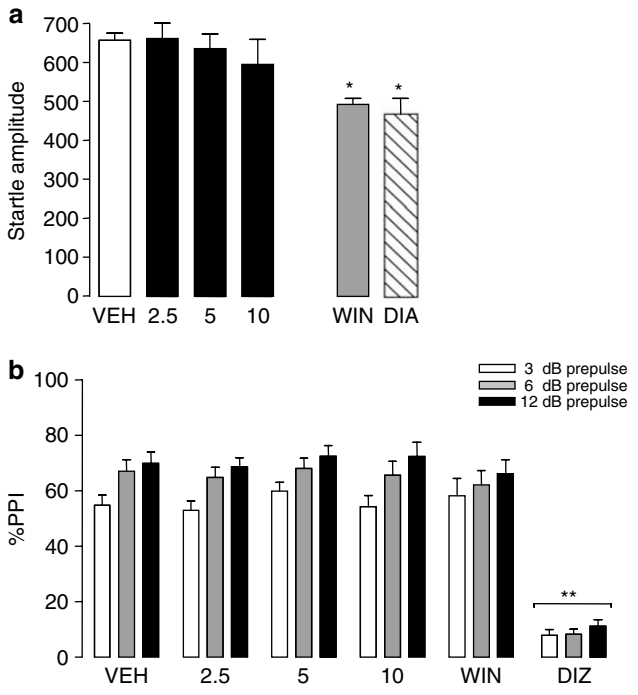

Figure 4 Effects of AM404 on (a) startle amplitude and (b) PPI in rats. (a) Effects of AM404 (2.5–10 mg kg^{-1}, i.p.), WIN55,212-2 (WIN, 1 mg kg^{-1}, i.p.) and diazepam (DIA, 5 mg kg^{-1}, i.p.) on startle reflex amplitude; *$p < 0.05$ compared with vehicle (VEH); (b) Effects of AM404 (2.5–10 mg kg^{-1}, i.p.), WIN55,212-2 (1 mg kg^{-1}, i.p.) and dizocilpine (DIZ, 0.1 mg kg^{-1}, s.c.) on PPI. AM404 and WIN55,212-2 were injected 45 min before testing, while diazepam and dizocilpine was injected 60 and 5 min before testing, respectively. Prepulse intensity is expressed in decibels above background noise. *$p < 0.05$, **$p < 0.01$ compared with vehicle; $n = 8$–11 per group.

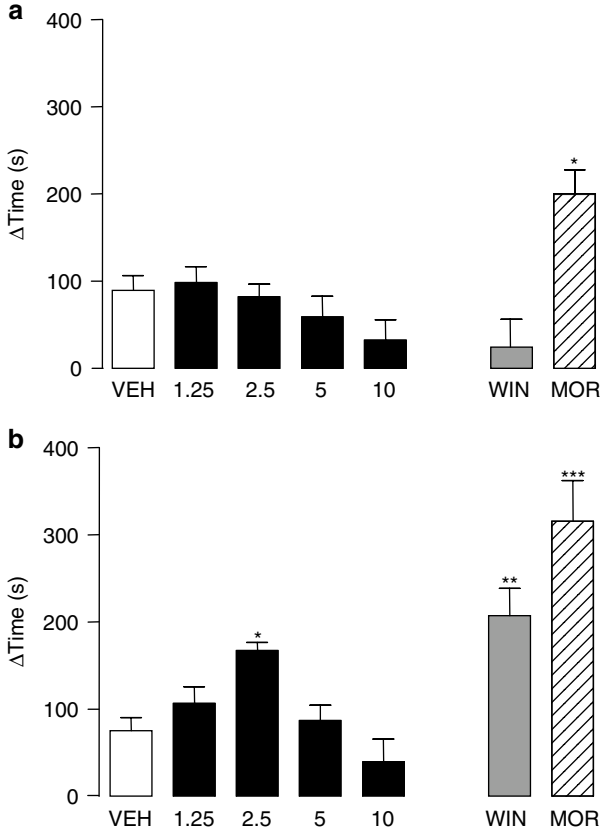

Figure 5 Effects of AM404 (1.25–10 mg kg^{-1}, i.p.), WIN55,212-2 (WIN, 1 mg kg^{-1}, i.p.), morphine (MOR, 5 mg kg^{-1}, s.c.) in the CPP test performed in rats housed under (a) enriched or (b) nonenriched conditions. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ in comparison with vehicle (VEH); $n = 8$–12 per group.

($F_{6,59} = 8.86$, $p < 0.001$ for main treatment effect; $p < 0.05$ for *post hoc* comparison between vehicle and AM404 2.5 mg kg^{-1} $p < 0.01$ for *post hoc* comparison between vehicle and WIN55,212-2; $p < 0.001$ for *post hoc* comparison between vehicle and morphine). Statistical analysis indicated, however, that the preference shift induced by 2.5 mg kg^{-1} AM404 was significantly lower than that elicited by 5 mg kg^{-1} morphine ($p < 0.001$, Tukey's test).

DISCUSSION

The present study shows that the endocannabinoid transport inhibitor AM404 selectively increases levels of anandamide, but not 2-AG, in the rat prefrontal cortex, hippocampus and thalamus, three brain regions that are intimately involved in the regulation of stress and emotion (Nestler *et al*, 2002; Cahill and McGaugh, 1998). This biochemical response is accompanied by marked anxiolytic-like effects, which are prevented by the CB_1 receptor antagonist rimonabant. In contrast, AM404 exerts only modest motivational effects in the CPP test and does not influence startle reflex or PPI of startle.

The anxiolytic-like effects elicited by AM404 and the sensitivity of these effects to rimonabant suggest that endogenously produced anandamide is involved in the regulation of anxiety, plausibly via activation of brain CB_1 receptors. Such a mechanism might also account for the antidepressant-like effects of AM404, which were recently documented using the rat forced-swim test (Hill and Gorzalka, 2005). Our results complement other lines of evidence suggesting a role for anandamide in the modulation of emotional responses to stress. Stressful stimuli affect anandamide mobilization in brain regions that are involved in the control of emotion. In rats, for example, a single electric shock to the paw elevates anandamide levels in the midbrain (Hohmann *et al*, 2005), while in mice physical restraint decreases anandamide levels in the amygdala (Patel *et al*, 2004). Moreover, pharmacological blockade or genetic ablation of CB_1 receptors exacerbates normal reactions to acute stress, presumably by disabling an endocannabinoid modulation of these reactions (Navarro *et al*, 1997; Haller *et al*, 2004; Urigüen *et al*, 2004). Finally, the FAAH inhibitor URB597 enhances stress-coping behaviors in a rimonabant-sensitive manner (Kathuria *et al*, 2003; Gobbi *et al*, 2005), suggesting that anandamide interacts with a subgroup of CB_1 receptors in the brain that regulate stress responses.

Brain CB_1 receptors are predominantly localized on axon terminals of γ-amino-butyric acid (GABA)ergic and glutamatergic neurons, and their activation inhibits the release of GABA and glutamate in the hippocampus, amygdala and other regions of the brain (for review, see Freund *et al*, 2003). Thus, the modulation of either inhibitory or excitatory transmitter systems may be involved in the regulation of emotional behavior by anandamide. In addition, anandamide might also influence the release of anxiogenic neuropeptides, such as corticotropin-releasing factor and cholecystokinin-octapeptide (Weidenfeld *et al*, 1994; Beinfeld and Connolly, 2001).

In contrast with previous experiments in mice (Fernandez-Espejo and Galan-Rodriguez, 2004), we found that AM404 does not affect startle reflex and PPI in Sprague–Dawley rats. Differences in animal species and experimental protocol are likely to account for this discrepancy. For example, those studies were performed in a local colony of Swiss mice, which did not show either loudness dependence in baseline PPI or dose dependence in PPI disruption induced by acute administration of AM404. Irrespective of possible explanations, our findings clearly indicate that blockade of anandamide transport has little impact on sensory reactivity in Sprague–Dawley rats. Moreover, since PPI disruption is considered to be a predictor of perceptual impairment and hallucinatory potential (Swerdlow *et al*, 2000), the results also suggest that AM404 may be devoid of acute psychotomimetic effects.

AM404 produced an inverse U-shaped response in the CPP paradigm, but only when administered to rats housed under enriched conditions. At the dose of 2.5 mg kg^{-1}, the effect of AM404 was similar to that elicited by the direct-acting cannabinoid agonist WIN55,212-2, albeit lower than that produced by morphine. Conversely, no response to either AM404 or WIN55,212-2 was observed in rats kept under nonenriched conditions. These results are consistent with those of other investigations, showing that environmental factors can greatly affect responses in the CPP test (Bowling and Bardo, 1994; Smith *et al*, 2003). As such, they might help interpret some of the discrepancies reported on the effects of cannabinoid agonists in this model (Gardner, 2005). In addition, our findings are consistent with recent findings indicating that both anandamide (Justinova *et al*, 2005) and AM404 are intravenously self-administered by squirrel monkeys (Justinova and Goldberg, 2005).

Previous reports have shown that AM404 does not closely mimic the spectrum of pharmacological responses produced by direct cannabinoid agonists, since it does not elicit catalepsy, acute antinociception or hypothermia (Beltramo *et al*, 1997, 2000; Fegley *et al*, 2004). These differences have been attributed to the ability of AM404 to inhibit endocannabinoid transport without directly activating CB_1 receptors (Beltramo *et al*, 1997, 2000). Recently, the existence of an endocannabinoid transport system has been questioned in favor of a simple diffusion mechanism, whereby anandamide accumulation may be solely driven by an inward concentration gradient maintained by FAAH-mediated hydrolysis (Glaser *et al*, 2003). In this context, the actions of AM404 have also been ascribed to its ability to serve as a FAAH substrate and to compete with anandamide for FAAH activity. However, the discovery that genetic deletion of FAAH does not affect anandamide internalization in neurons argues against this possibility and in favor of the transporter hypothesis (Fegley *et al*, 2004; Alger, 2004; Ortega-Gutierrez *et al*, 2004). Additional support to this hypothesis comes from the recent discovery of potent nonaliphatic inhibitors of endocannabinoid transport, which has led to the identification of a high-affinity binding site presumably involved in the transport process (Moore *et al*, 2005). Noteworthy, the effects produced by AM404 are markedly different from those exerted by the FAAH inhibitor URB597 in at least two respects. First, AM404 does not affect brain levels of OEA and PEA, which are enhanced by URB597 (Kathuria *et al*, 2003; Fegley *et al*, 2005). Second, although AM404 mirrors the anxiolytic-like effects of URB597 (Kathuria *et al*, 2003), the

latter does not exert any significant motivational effect in the CPP model, irrespective of housing conditions (Gobbi *et al*, 2005).

In addition to its inhibitory actions on anandamide transport, AM404 interacts *in vitro* with several unrelated pharmacological targets, such as vanilloid TRPV1 receptors (Zygmunt *et al*, 2000) and sodium channels (Nicholson *et al*, 2003). While the *in vivo* relevance of these effects is still unclear, we cannot rule out that they might contribute to the pharmacological properties of AM404. Nevertheless, the ability of selective doses of the CB_1 receptor antagonist rimonabant to prevent the anxiolytic-like actions of AM404 suggests that such actions can be ascribed to anandamide-mediated activation of CB_1 receptors. To date, AM404 remains the best characterized among a small series of endocannabinoid transport inhibitors that have been developed. Indeed, the compound AM1172 (Fegley *et al*, 2004) also acts as a direct partial agonist of CB_1 receptors, while other agents, such as VDM11, have been shown to interact with multiple targets (Kelley and Thayer, 2004). UCM707 induces hypomotility and antinociception (de Lago *et al*, 2002), but its impact on behavior is still incompletely documented. Thus, the compounds mentioned above offer little or no advantage over AM404 for *in vivo* studies. The recent availability of new anandamide transport inhibitors, such as LY2183240 (Moore *et al*, 2005), will allow a more detailed characterization of this target in the future. In conclusion, while our results point to the anandamide transport system as a target for anxiolytic drugs, they also highlight the need to fully characterize this system at the molecular level, and to develop more advanced probes to validate its therapeutic potential.

ACKNOWLEDGEMENTS

The present study was supported by grants from National Institute on Drug Abuse (DA-12447 and DA-3412) and the National Alliance for Research on Schizophrenia and Depression (to DP), and Ministero dell'Istruzione, Università e Ricerca (to VC and GLG). MB was a NIDA INVEST fellow. PC was an Italian Society of Pharmacology fellow. We thank Oliver Arguello and Marco Orrù for experimental assistance.

REFERENCES

Abduljawad KA, Langley RW, Bradshaw CM, Szabadi E (1997). Effects of clonidine and diazepam on the acoustic startle response and on its inhibition by 'prepulses' in man. *J Psychopharmacol* **11**: 29–34.

Alger BE (2004). Endocannabinoids: getting the message across. *Proc Natl Acad Sci USA* **101**: 8512–8513.

Azad SC, Monory K, Marsicano G, Cravatt BF, Lutz B, Zieglgansberger W *et al* (2004). Circuitry for associative plasticity in the amygdala involves endocannabinoid signaling. *J Neurosci* **24**: 9953–9961.

Bahri T, Amir T (1994). Effect of hashish on vigilance performance. *Percept Mot Skills* **78**: 11–16.

Beinfeld MC, Connolly K (2001). Activation of CB1 cannabinoid receptors in rat hippocampal slices inhibits potassium-evoked cholecystokinin release, a possible mechanism contributing to the spatial memory defects produced by cannabinoids. *Neurosci Lett* **301**: 69–71.

Beltramo M, de Fonseca FR, Navarro M, Calignano A, Gorriti MA, Grammatikopoulos G *et al* (2000). Reversal of dopamine D(2). receptor responses by an anandamide transport inhibitor. *J Neurosci* **20**: 3401–3407.

Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A, Piomelli D (1997). Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science* **277**: 1094–1097.

Bortolato M, Aru GN, Frau R, Orru M, Luckey GC, Boi G *et al* (2005). The CB receptor agonist WIN 55,212-2 fails to elicit disruption of prepulse inhibition of the startle in Sprague–Dawley rats. *Psychopharmacology* **177**: 264–271.

Bowling SL, Bardo MT (1994). Locomotor and rewarding effects of amphetamine in enriched, social, and isolate reared rats. *Pharmacol Biochem Behav* **48**: 459–464.

Cagiano R, Barfield RJ, White NR, Pleim ET, Weinstein M, Cuomo V (1988). Subtle behavioural changes produced in rat pups by in utero exposure to haloperidol. *Eur J Pharmacol* **157**: 45–50.

Cahill L, McGaugh JL (1998). Mechanisms of emotional arousal and lasting declarative memory. *Trends Neurosci* **21**: 294–299.

Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR *et al* (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci USA* **98**: 9371–9376.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384**: 83–87.

D'Souza DC, Abi-Saab WM, Madonick S, Forselius-Bielen K, Doersch A, Braley G *et al* (2005). Delta-9-tetrahydrocannabinol effects in schizophrenia: implications for cognition, psychosis, and addiction. *Biol Psychiatr* **57**: 594–608.

de Lago E, Fernandez-Ruiz J, Ortega-Gutierrez S, Viso A, Lopez-Rodriguez ML, Ramos JA (2002). UCM707, a potent and selective inhibitor of endocannabinoid uptake, potentiates hypokinetic and antinociceptive effects of anandamide. *Eur J Pharmacol* **449**: 99–103.

Désarnaud F, Cadas H, Piomelli D (1995). Anandamide amidohydrolase activity in rat brain microsomes. Identification and partial characterization. *J Biol Chem* **270**: 6030–6035.

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC *et al* (1994). Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372**: 686–691.

Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G *et al* (2005). Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester (URB597). Effects on anandamide and oleoylethanolamide deactivation. *J Pharmacol Exp Ther* **313**: 352–358.

Fegley D, Kathuria S, Mercier R, Li C, Goutopoulos A, Makriyannis A *et al* (2004). Anandamide transport is independent of fatty-acid amide hydrolase activity and is blocked by the hydrolysis-resistant inhibitor AM1172. *Proc Natl Acad Sci USA* **101**: 8756–8761.

Fernandez-Espejo E, Galan-Rodriguez B (2004). Sensorimotor gating in mice is disrupted after AM404, an anandamide reuptake and degradation inhibitor. *Psychopharmacology* **175**: 220–224.

Freund TF, Katona I, Piomelli D (2003). Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev* **83**: 1017–1066.

Gardner EL (2005). Endocannabinoid signaling system and brain reward: emphasis on dopamine. *Pharmacol Biochem Behav* **81**: 263–284.

Gerdeman GL, Ronesi J, Lovinger DM (2002). Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. *Nat Neurosci* **5**: 446–451.

Gifford AN, Bruneus M, Gatley SJ, Lan R, Makriyannis A, Volkow ND (1999). Large receptor reserve for cannabinoid actions in the central nervous system. *J Pharmacol Exp Ther* **288**: 478–483.

Giuffrida A, Parsons LH, Kerr TM, Rodriguez de Fonseca F, Navarro M, Piomelli D (1999). Dopamine activation of

endogenous cannabinoid signaling in dorsal striatum. *Nat Neurosci* **2**: 358–363.

Glaser ST, Abumrad NA, Fatade F, Kaczocha M, Studholme KM, Deutsch DG (2003). Evidence against the presence of an anandamide transporter. *Proc Natl Acad Sci USA* **100**: 4269–4274.

Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M *et al* (2005). Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proc Natl Acad Sci USA* **102**: 18620–18625.

Griebel G, Simiand J, Serradeil-Le Gal C, Wagnon J, Pascal M, Scatton B *et al* (2002). Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V1b receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. *Proc Natl Acad Sci USA* **99**: 6370–6375.

Haller J, Varga B, Ledent C, Freund TF (2004). CB1 cannabinoid receptors mediate anxiolytic effects: convergent genetic and pharmacological evidence with CB1-specific agents. *Behav Pharmacol* **15**: 299–304.

Hill MN, Gorzalka BB (2005). Pharmacological enhancement of cannabinoid CB (1) receptor activity elicits an antidepressant-like response in the rat forced swim test. *Eur Neuropsychopharmacol* **15**: 593–599.

Hillard CJ, Edgemond WS, Campbell WB (1995). Characterization of ligand binding to the cannabinoid receptor of rat brain membranes using a novel method: application to anandamide. *J Neurochem* **64**: 677–683.

Hillard CJ, Edgemond WS, Jarrahian A, Campbell WB (1997). Accumulation of *N*-arachidonoylethanolamine (anandamide). into cerebellar granule cells occurs via facilitated diffusion. *J Neurochem* **69**: 631–638.

Hillard CJ, Jarrahian A (2003). Cellular accumulation of anandamide: consensus and controversy. *Br J Pharmacol* **140**: 802–808.

Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley DB, Mangieri RA *et al* (2005). An endocannabinoid mechanism for stress-induced analgesia. *Nature* **435**: 1108–1112.

Hollister LE (1998). Health aspects of cannabis: revisited. *Int J Neuropsychopharmacol* **1**: 71–80.

Justinova Z, Goldberg SR (2005). The abuse potential of the endocannabinoid transport inhibitor AM404: self-administration by squirrel monkeys. 15th Annual Symposium on the cannabinoids, 2005, Clearwater, USA.

Justinova Z, Solinas M, Tanda G, Redhi GH, Goldberg SR (2005). The endogenous cannabinoid anandamide and its synthetic analog R(+)-methanandamide are intravenously self-administered by squirrel monkeys. *J Neurosci* **25**: 5645–5650.

Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A *et al* (2003). Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* **9**: 76–81.

Kelley BG, Thayer SA (2004). Anandamide transport inhibitor AM404 and structurally related compounds inhibit synaptic transmission between rat hippocampal neurons in culture independent of cannabinoid CB1 receptors. *Eur J Pharmacol* **496**: 33–39.

Koelega HS (1989). Benzodiazepines and vigilance performance: a review. *Psychopharmacology* **98**: 145–156.

Larsson F, Winblad B, Mohammed AH (2002). Psychological stress and environmental adaptation in enriched *vs* impoverished housed rats. *Pharmacol Biochem Behav* **73**: 193–207.

Lichtman AH, Leung D, Shelton CC, Saghatelian A, Hardouin C, Boger DL *et al* (2004). Reversible inhibitors of fatty acid amide hydrolase that promote analgesia: evidence for an unprecedented combination of potency and selectivity. *J Pharmacol Exp Ther* **311**: 441–448.

Moore SA, Nomikos GG, Dickason-Chesterfield AK, Schober DA, Schaus JM, Ying BP *et al* (2005). Identification of a high-affinity binding site involved in the transport of endocannabinoids. *Proc Natl Acad USA* **102**: 17852–17857.

Navarro M, Hernandez E, Munoz RM, del Arco I, Villanua MA, Carrera MR *et al* (1997). Acute administration of the CB1 cannabinoid receptor antagonist SR 141716A induces anxiety-like responses in the rat. *Neuroreport* **8**: 491–496.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM (2002). Neurobiology of depression. *Neuron* **34**: 13–25.

Nicholson RA, Liao C, Zheng J, David LS, Coyne L, Errington AC *et al* (2003). Sodium channel inhibition by anandamide and synthetic cannabimimetics in brain. *Brain Res* **978**: 194–204.

Ortega-Gutierrez S, Hawkins EG, Viso A, Lopez-Rodriguez ML, Cravatt BF (2004). Comparison of anandamide transport in FAAH wild-type and knockout neurons: evidence for contributions by both FAAH and the CB1 receptor to anandamide uptake. *Biochemistry* **43**: 8184–8190.

Palmer SL, Thakur GA, Makriyannis A (2002). Cannabinergic ligands. *Chem Phys Lipids* **121**: 3–19.

Patel S, Roelke CT, Rademacher DJ, Cullinan WE, Hillard CJ (2004). Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic–pituitary–adrenal axis. *Endocrinology* **145**: 5431–5438.

Pellow S, Chopin P, File SE, Briley M (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* **14**: 149–167.

Piomelli D (2005). The endocannabinoid system: a drug discovery perspective. *Curr Opin Investig Drugs* **6**: 672–679.

Piomelli D, Beltramo M, Glasnapp S, Lin SY, Goutopoulos A, Xie XQ *et al* (1999). Structural determinants for recognition and translocation by the anandamide transporter. *Proc Natl Acad Sci USA* **96**: 5802–5807.

Renner MJ, Rosenzweig MR (1987). The golden-mantled ground squirrel (*Spermophilus lateralis*). As a model for the effects of environmental enrichment in solitary animals. *Dev Psychobiol* **20**: 19–24.

Smith MA, Bryant PA, McClean JM (2003). Social and environmental enrichment enhances sensitivity to the effects of kappa opioids: studies on antinociception, diuresis and conditioned place preference. *Pharmacol Biochem Behav* **76**: 93–101.

Steffens M, Szabo B, Klar M, Rominger A, Zentner J, Feuerstein TJ (2003). Modulation of electrically evoked acetylcholine release through cannabinoid CB1 receptors: evidence for an endocannabinoid tone in the human neocortex. *Neuroscience* **120**: 455–465.

Swerdlow NR, Braff DL, Geyer MA (2000). Animal models of deficient sensorimotor gating: what we know, what we think we know, and what we hope to know soon. *Behav Pharmacol* **11**: 185–204.

Takahashi LK, Ho SP, Livanov V, Graciani N, Arneric SP (2001). Antagonism of CRF2 receptors produces anxiolytic behavior in animal models of anxiety. *Brain Res* **902**: 135–142.

Tzavara ET, Wade M, Nomikos GG (2003). Biphasic effects of cannabinoids on acetylcholine release in the hippocampus: site and mechanism of action. *J Neurosci* **23**: 9374–9384.

Ueda N, Kurahashi Y, Yamamoto S, Tokunaga T (1995). Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. *J Biol Chem* **270**: 23823–23827.

Urigüen L, Perez-Rial S, Ledent C, Palomo T, Manzanares J (2004). Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB1 receptors. *Neuropharmacology* **46**: 966–973.

Weidenfeld J, Feldman S, Mechoulam R (1994). Effect of the brain constituent anandamide, a cannabinoid receptor agonist, on the hypothalamo–pituitary–adrenal axis in the rat. *Neuroendocrinology* **59**: 110–112.

Zygmunt PM, Chuang H, Movahed P, Julius D, Hogestatt ED (2000). The anandamide transport inhibitor AM404 activates vanilloid receptors. *Eur J Pharmacol* **396**: 39–42.