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Bortolato, Marco
Mangieri, Regina A
Fu, Jin
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Antidepressant-like Activity of the Fatty Acid Amide Hydrolase Inhibitor URB597 in a Rat Model of Chronic Mild Stress

Marco Bortolato, Regina A. Mangieri, Jin Fu, Janet H. Kim, Oliver Arguello, Andrea Duranti, Andrea Tontini, Marco Mor, Giorgio Tarzia, and Daniele Piomelli

Background: The endocannabinoid anandamide may be involved in the regulation of emotional reactivity. In particular, it has been shown that pharmacological inhibition of the enzyme fatty acid amide hydrolase (FAAH), which catalyzes the intracellular hydrolysis of anandamide, elicits anxiolytic-like and antidepressant-like effects in rodents.

Methods: We investigated the impact of chronic treatment with the selective FAAH inhibitor, URB597 (also termed KDS-4103), on the outcomes of the chronic mild stress (CMS) in rats, a behavioral model with high isomorphism to human depression.

Results: Daily administration of URB597 (.3 mg·kg^{-1}, intraperitoneal [IP]) for 5 weeks corrected the reduction in body weight gain and sucrose intake induced by CMS. The antidepressant imipramine (20 mg·kg^{-1}, once daily, IP) produced a similar response, whereas lower doses of URB597 were either marginally effective (.1 mg·kg^{-1}) or ineffective (.03 mg·kg^{-1}). Treatment with URB597 (.3 mg·kg^{-1}) resulted in a profound inhibition of brain FAAH activity in both CMS-exposed and control rats. Furthermore, the drug regimen increased anandamide levels in midbrain, striatum, and thalamus.

Conclusions: URB597 exerts antidepressant-like effects in a highly specific and predictive animal model of depression. These effects may depend on the ability of URB597 to enhance anandamide signaling in select regions of the brain.

Cannabinoid receptors, the molecular target of Δ^9-tetrahydrocannabinol (Δ^9-THC), are physiologically engaged by a family of arachidonic acid derivatives, the endocannabinoids, which include anandamide and 2-arachidonoylglycerol (2-AG) (Devane *et al.* 1992; Di Marzo *et al.* 1994; Mechoulam *et al.* 1995; Stella *et al.* 1997; Sugiura *et al.* 1995). After release, anandamide is rapidly removed from the extracellular space by high-affinity, carrier-mediated transport (Beltramo *et al.* 1997; Hillard and Campbell 1997; Fegley *et al.* 2004; Hillard and Jarrahian 2000) and is subsequently hydrolyzed within cells by the enzyme fatty acid amide hydrolase (FAAH), a serine hydrolase that also cleaves the noncannabinoid lipid amides oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) (McKinney and Cravatt 2005).

Various classes of FAAH inhibitors have been developed (Piomelli 2005). Among them is a group of *O*-aryl-carbamates that inhibit FAAH activity with high potency and selectivity (Fegley *et al.* 2005; Kathuria *et al.* 2003; Mor *et al.* 2004; Tarzia *et al.* 2003). A well-characterized member of this class, the compound URB597 (also termed KDS-4103), blocks rat brain FAAH activity in vivo with a half-maximal inhibitory dose (ID$_{50}$) of .15 mg·kg^{-1} (intraperitoneal [IP]), increases brain anandamide levels, and enhances the pharmacological effects of exogenous anandamide (Fegley *et al.* 2005; Kathuria *et al.* 2003). URB597 does not elicit a typical spectrum of cannabinoid responses (Kathuria *et al.* 2003)—for example, it does not produce catalepsy, hypothermia, or hyperphagia (Fegley *et al.* 2005)—but does exert significant analgesic, anxiolytic-like, and antidepressant-like effects. For example, it reduces nocifensive behavior in rodent models of inflammatory pain (Holt *et al.* 2005; Jayamanne *et al.* 2006), increases the amount of time spent by rats and mice in the open arms of an elevated maze (Kathuria *et al.* 2003; Patel and Hillard 2006), lowers isolation-induced vocalizations in rat pups (Kathuria *et al.* 2003), and decreases plasma corticosterone levels in restrained mice (Patel *et al.* 2004). In addition, URB597 enhances stress-coping responses in the rat forced swim test and the mouse tail suspension test (Gobbi *et al.* 2005). Notably, these effects are not accompanied by overt rewarding actions, as suggested by the inability of URB597 to induce conditioned place preference or substitute for Δ^9-THC in a drug-discrimination paradigm (Gobbi *et al.* 2005). Thus, unlike Δ^9-THC and other direct-acting cannabinoid receptor agonists (Lichtman and Martin 2005), URB597 appears to be devoid of significant abuse potential.

The effects of URB597 on stress-coping behaviors are consistent with evidence indicating the involvement of endocannabinoids in the pathophysiology of depression. Thus, pharmacological blockade of endocannabinoid transport elicits antidepressant-like responses in the rat forced swim test (Hill and Gorzalka 2005). Moreover, cannabinoid-1 (CB1) deficient mice display a series of behavioral alterations related to human depressive symptoms, including anhedonia, anxiety, enhanced stress responsiveness, and feeding dysregulation (Cota *et al.* 2003; Martin *et al.* 2002; Sanchis-Segura *et al.* 2004).

Acute stress based models, such as the forced swim and the tail suspension tests, are inadequate to investigate the neurobiological relationships between chronic stress and the pathogenesis of mood disorders and fail to reproduce the constellation of symptoms observed in depression. An experimental paradigm

that mimics more closely several of such symptoms is chronic mild stress (CMS) (Willner *et al*. 1987). This model is based on the finding that rodents exposed to an unpredictable, relatively continuous sequence of mild stressors develop a series of abnormal behavioral and physiological responses that are reminiscent of those observed in depressed patients (DSM-IV 296.2X–296.3X [American Psychiatric Association 1994]): decreased responsiveness to rewarding stimuli such as palatable foods, alterations in feeding and body weight (BW), enhanced fearfulness, impaired sleep architecture, and inadequate self-care (Willner 2005). Importantly, these abnormalities are corrected by chronic, but not acute, administration of antidepressant drugs. For these reasons, CMS is currently regarded by many investigators as one of the most naturalistic and predictive animal models of depression (Henn and Vollmayr 2005; Willner 1997; Willner 2005).

In the present study, we examined the behavioral impact of URB597 in rats exposed to CMS. We monitored changes in body weight and sucrose intake as indices of stress-induced responses, since both parameters are consistently reduced by CMS in an antidepressant-sensitive manner (Moreau 1997; Willner 1997).

Methods and Materials

Subjects

One hundred twenty male Wistar rats (Charles River, Raleigh, North Carolina), weighing approximately 200 g upon arrival at the animal facilities, served as subjects in the present study. They were individually housed in rooms maintained on a 12-hour light-dark cycle (lights on at 7:00 AM and off at 7:00 PM) under equivalent conditions of temperature (22 ± 2°C) and relative humidity (55% to 60%) Food and water were available ad libitum, except during the periods of food and water deprivation required by the stress protocol or before assessment of sucrose consumption. All experimental procedures were approved by the local ethics committee and carried out in strict accordance with the National Institutes of Health guidelines for care and use of experimental animals.

Drugs

URB597 was obtained from Kadmus Pharmaceuticals (Irvine, California) and the Institute of Medicinal Chemistry, University of Urbino "Carlo Bo" (Italy). The compound was prepared for administration as follows: 3 mg was suspended in .5 mL polyethylene glycol (PEG-400) (Sigma-Aldrich, St. Louis, Missouri), vigorously stirring the mixture with a spatula; .5 mL Tween-80 (Sigma-Aldrich) was added, and the mixture was sonicated for 5 min, obtaining a clear solution. Nine milliliters of .9% saline were added and the resulting suspension was sonicated for 10 min at room temperature. Thus, the final drug concentration was .3 mg/mL in a vehicle of PEG-400, Tween-80, and saline (5:5:90, vol/vol). Imipramine hydrochloride was purchased from Sigma-Aldrich and dissolved in saline with a few drops of .1 mol/L sodium hydroxide (NaOH) to normalize pH.

Experimental Procedures

Sucrose Consumption. Upon arrival, the rats were randomly assigned to one of two separate rooms (60 rats per room), where they were housed in individual cages. After an initial habituation period of 10 days, all animals were tested for baseline sucrose consumption. They were deprived of food and water for 15 hours prior to the test, starting 1 hour before the onset of the dark phase. Each animal was given access to one preweighed bottle containing a 1% sucrose solution in tap water. One hour later, the

bottle was removed and weighed again, and food and water were placed back in the cage. Sucrose consumption tests were repeated every 3 to 4 days for the following 2 weeks. After stabilization of sucrose consumption, baseline sucrose preference was assessed. The preference test was similar to the consumption test, except that the animals were given the choice between a 1% sucrose solution and plain water. After preference assessment, each group was divided into five matched subgroups (*n* = 12 each) with sucrose consumption and preference equally distributed across subgroups. The preference test was run only once, immediately before the beginning of the CMS protocol.

CMS Protocol. In one of the two rooms, rats were subjected to a random sequence of mild stressors (Willner *et al*. 1987). These included cage soiling with water, group housing in a confined space, water and/or food deprivation, intermittent lighting, reversal of light/dark cycle, cage tilting to 45°, exposure to loud white noise, and strobe lights (300 flashes/min). Stressors were individually applied in succession and lasted 8 to 12 hours each. Control rats housed in the second room were not subjected to the stress protocol but were food and water deprived for a period of 15 hours preceding sucrose consumption tests like CMS-exposed rats. Sucrose intake and body weight were measured weekly for the duration of the study.

Drug Treatments. Starting 5 weeks after the beginning of the CMS protocol, control and CMS-exposed rats received daily injections of vehicle, URB597 (.03, .1, .3 mg·kg^{-1}, IP), or imipramine (20 mg·kg^{-1}, IP). All drugs were freshly prepared and administered in 1 mL·kg^{-1} of vehicle. All the injections were made between 4:00 PM and 6:00 PM, irrespective of the stress schedule.

Lipid Analyses. At the end of the study, the rats were anesthetized with halothane and decapitated during the application of the last stressor (2 hours after the last drug or vehicle injection). Brains were removed within approximately 30 sec of decapitation and select regions were dissected using a rat brain atlas (Paxinos and Watson 1986) as a guide, frozen in dry ice, and stored at -80°C until analysis. Endocannabinoids and related lipids were extracted with methanol-chloroform, fractionated by open-bed silica gel chromatography, and quantified by isotope-dilution liquid chromatography/mass spectrometry (LC/MS), as described by Fegley *et al*. (2005).

FAAH Assay. Fatty acid amide hydrolase activity was assayed using [³H]-anandamide (arachidonoyl [ethanolamide-³H]) (American Radiolabeled Chemicals, ARC, St. Louis, Missouri; 60 Ci/mmol) as a substrate. Brain region homogenates (50 μg protein) were incubated with [³H]-anandamide (10,000 disintregrations per minute [dpm]) for 30 minutes at 37°C in a total volume of .5 mL of 50 mmol/L Tris-HCl buffer (pH 7.4) (Sigma-Aldrich) containing .05% fatty acid-free bovine serum albumin. The reactions were terminated by adding 1 mL of chloroform-methanol (1:1, vol/vol) and separated by centrifugation at 1000*g* for 10 min. The aqueous layer was collected and [³H]ethanolamine was measured in the aqueous phase by liquid scintillation counting.

Real-Time Quantitative Polymerase Chain Reaction. Total RNA was extracted from midbrain tissues with TRIzol (Invitrogen, Carlsbad, California). Complementary DNA (cDNA) was synthesized with a custom kit (Stratagene, La Jolla, California) following the manufacturer's instructions. Real-time quantitative (RTQ) polymerase chain reaction (PCR) was performed using a Mx 3000P system (Stratagene). Primers and fluorogenic probes were synthesized at TIB (Adelphia, New Jersey). Primer/probe sequences

were: FAAH, forward primer: GCCTCAAGGAATG CTTCAGC, reverse primer: TGCCCTCATTCAGGCTCAAG, probe: ACAAGGG CCACGACTCCACACTGG; CB1, forward primer: CACAAGCACGC-CAATAA CACA, reverse primer: ACAGTGCTCTTGATGCAGCTTTC, probe: CCAGCATGCACAGGGCCGC; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward primer: AAGTATGATGACATC AAGAAGGTGGT, reverse primer: AGCCCAGGATGCCCTTAG, probe: AAG CAGGCGGCCGAGGGC. Messenger RNA (mRNA) levels were normalized by using GAPDH as an internal standard and were measured as described (Halford 1999).

Data Analysis

Results were analyzed using multifactor analysis of variance (MANOVA), followed by Spjotvoll-Stoline correction for Tukey's test for post hoc comparisons. Multivariate comparisons for repeated measures were analyzed with the Dunn-Sidak correction for Bonferroni test. Throughout the study, the impact of CMS was studied calculating absolute sucrose intake (ASI) and body weight variations. To test whether changes in sucrose intake could be affected by body weight alterations, we also analyzed differences in relative sucrose consumption, defined as the ratio between absolute sucrose intake and body weight (Bekris *et al.* 2005; Willner *et al.* 1996). We used the Pearson product-moment correlation coefficient to examine the correlation between individual differences in absolute sucrose intake and body weight between the onset of the CMS protocol and treatment initiation.

Results

Effects of CMS on Body Weight Gain and Sucrose Intake in Rats

Figure 1 illustrates the effects of CMS on body weight, absolute sucrose intake, and relative sucrose intake (RSI) in the two groups of animals used in the present study ($n = 60$ each).
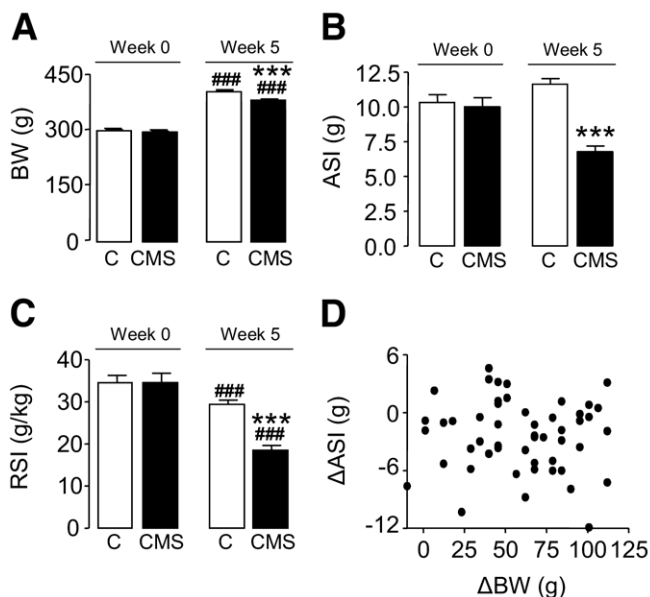
Figure 1. Effects of CMS on **(A)** body weight (BW), **(B)** absolute sucrose intake (ASI), and **(C)** relative sucrose intake (RSI); **(D)** comparison of individual variations in ASI and BW between the beginning and the fifth week of CMS. Values are expressed as mean ± SEM. C, control rats; CMS, CMS-exposed rats. ***$p < .001$ versus control rats; ###$p < .01$ in comparison with week 0. CMS, chronic mild stress; BW, body weight; ASI, absolute sucrose intake; RSI, relative sucrose intake.
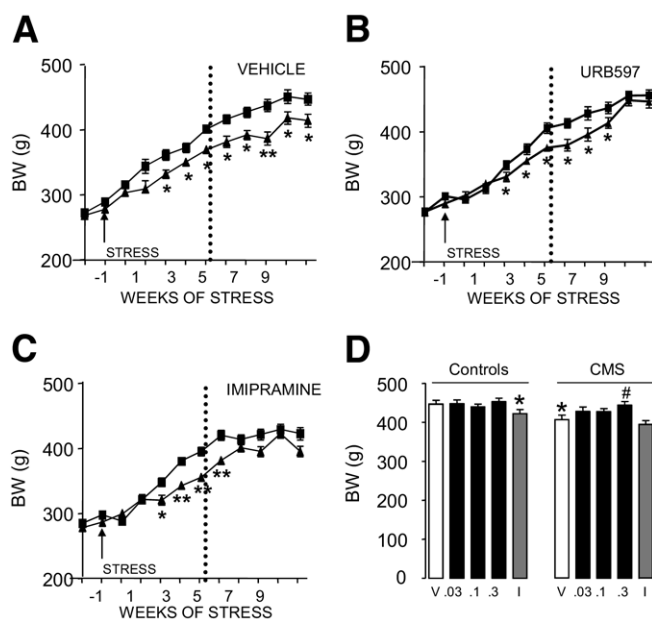

Figure 2. Effects of **(A)** vehicle, **(B)** URB597 (.3 mg·kg^{-1}, IP), and **(C)** imipramine (20 mg·kg^{-1}, IP) on BW of CMS-exposed and control rats; **(D)** comparison of effects of vehicle, URB597, and imipramine in CMS-exposed and control rats at the 10th week. Values are expressed as mean ± SEM ■, control rats; ▲, CMS-exposed rats. *$p < .05$ versus control rats; **$p < .01$ versus control rats; #$p < .05$ versus CMS-exposed, vehicle-treated rats. IP, intraperitoneal; BW, body weight; CMS, chronic mild stress.

Before starting the CMS protocol, the animals exhibited equivalent BW [$F(1,116) = 1.95$, ns], ASI [$F(1,111) = .14$, ns], and RSI [$F(1,111) = .14$, ns]. Five weeks after protocol inception, these three parameters were significantly lower in rats exposed to CMS than in control rats (Figure 1A–C) [BW: $F(1,117) = 45.13$, $p < .0001$; ASI: $F(1,108) = 70.51$, $p < .0001$; RSI: $F(1,108) = 52.31$, $p < .0001$]. In agreement with previous reports (Willner 1997), CMS-exposed animals weighed approximately 6% less than control rats (Figure 1A) (control rats: 372.1 ± 2.73 g; CMS: 347.9 ± 2.65 g). Moreover, a 41.9% decrement in ASI (Figure 1B) (control rats: 11.64 ± .39 g; CMS: 6.76 ± .43 g) and a 36.9% decrement in RSI (Figure 1C) (control rats: 29.41 ± 1.01 g/kg; CMS: 18.56 ± 1.11 g/kg) were observed in CMS-exposed rats compared with control rats. Analysis of the Pearson product-moment correlation coefficient revealed no significant correlation between CMS-induced changes in BW and ASI (Figure 1D) ($n = 51$; $r = -.03$, ns), indicating that the loss in BW does not account for the decrease in ASI. Reductions in BW gain were significant within 3 weeks of CMS initiation (Figure 2) [treatment: $F(1,117) = 83.51$, $p < .0001$; time: $F(4,468) = 235.19$, $p < .0001$; interaction treatment × time: $F(4,468) = 5.95$, $p < .0001$, MANOVA; $p < .001$ for comparison between weeks 0 and 3–5, Dunn-Sidak].

Effects of URB597 on Body Weight Gain

The effects of vehicle, URB597 (.3 mg·kg^{-1}), and imipramine (20 mg·kg^{-1}) on BW gain are shown in Figure 2. Daily vehicle administration did not affect the BW trajectories of control and CMS-exposed rats (Figure 2A) [interaction treatment × time in CMS-exposed rats: $F(40,490) = 2.85$ $p < .0001$, MANOVA; $p < .05$ for comparisons between control-vehicle and CMS-vehicle for weeks 3–10, Dunn-Sidak]. By contrast, URB597 administration normalized BW gain in CMS-exposed rats, bringing it to control levels within 4 weeks of treatment (Figure 2B) ($p < .05$
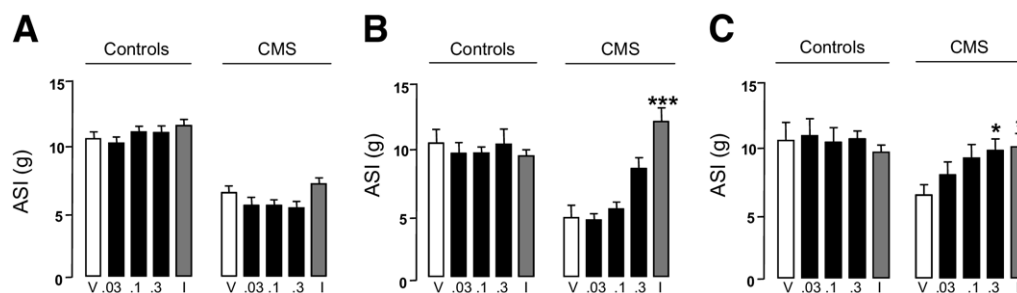
Figure 3. Effects of vehicle, URB597, and imipramine (20 mg·kg^{-1}, IP) on ASI in CMS-exposed and control rats at the end of the **(A)** second, **(B)** fourth, and **(C)** fifth week of treatment. Values are expressed as mean ± SEM. V, vehicle; I, imipramine. URB597 doses are expressed in mg·kg^{-1}. *$p < .05$ versus vehicle; ***$p < .001$ versus vehicle. IP, intraperitoneal; ASI, absolute sucrose intake; CMS, chronic mild stress.

for comparisons between control-URB597 .3 and CMS-URB597 .3 for weeks 3–8, Dunn-Sidak). Imipramine exerted an effect similar to, albeit somewhat faster than, URB597 (Figure 2C) ($p < .05$ for comparisons between control-imipramine and CMS-imipramine for weeks 3–6, Dunn-Sidak). Lower doses of URB597 (.03 and .1 mg·kg^{-1}) were ineffective (Figure 2D). In control rats, URB597 did not produce BW changes (Figure 2B, 2D) [interaction treatment × time in control rats: $F(40,530) = 1.37$, ns, two-way analysis of variance (ANOVA) for repeated measures], while imipramine caused a significant BW decrease (Figure 2D) [$F(1,21) = 4.40$, $p < .05$]. These findings suggest that URB597 corrects the loss in BW gain caused by CMS without affecting BW gain in nonstressed rats.

### Effects of URB597 on Absolute and Relative Sucrose Intake

Figure 3 depicts the effects of vehicle, URB597 (.03–.3 mg·kg^{-1}), and imipramine (20 mg·kg^{-1}) on ASI. After 5 weeks of treatment, URB597 (.3 mg·kg^{-1}) reversed the CMS-induced reduction in ASI (Figure 3C) [$F(4,39) = 4.13$, $p < .01$; $p < .05$ for comparisons between vehicle and URB597, Spjotvoll-Stoline]. Lower doses of URB597 showed a similar trend but did not reach statistical significance (Figure 3C). Imipramine exerted an effect quantitatively comparable with that of URB597 ($p < .05$ for comparisons between vehicle and URB597, Spjotvoll-Stoline), which was significant starting from the fourth week of treatment (Figure 3B, 3C) [$F(4,40) = 14.33$, $p < .0001$; $p < .001$ for comparisons between vehicle and imipramine, Spjotvoll-Stoline]. Neither URB597 nor imipramine altered ASI in control animals [fourth week of treatment: $F(4,53) = .45$, ns; fifth week of treatment: $F(4,53) = .20$, ns]. Although the BW increase was accompanied by a progressive reduction of RSI in all groups (Figure 4), this effect was significantly increased by CMS 2 weeks after protocol inception (Figure 4) [treatment: $F(1,108) = 150.15$, $p < .0001$; time: $F(4,432) = 43.07$, $p < .0001$; interaction treatment × time: $F(4,432) = 9.25$, $p < .0001$, MANOVA; $p < .001$ for comparisons between week 0 and weeks 2–5, Dunn-Sidak]. URB597 (.3 mg·kg^{-1}) and imipramine (20 mg·kg^{-1}) reversed this effect by the fourth week of treatment [$F(16,148) = 3.22$, $p < .0001$, MANOVA; $p < .05$ for comparisons between URB597 and vehicle and imipramine and vehicle at the first and second week; Spjotvoll-Stoline]. By contrast, neither URB597 nor imipramine affected RSI in control animals (Figure 4) [interaction treatment × time: $F(16,208) = .46$, ns]. We interpret these results to indicate that URB597 normalizes sucrose consumption in CMS-exposed rats but does not influence this parameter in nonstressed rats.
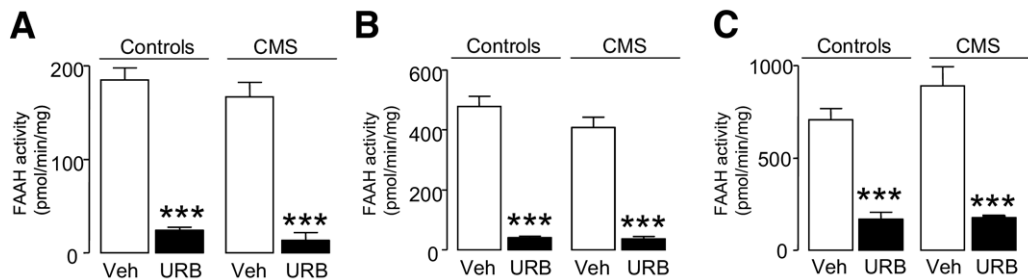
Effects of URB597 Treatment on FAAH Activity

The inhibitory effects of acute or subchronic URB597 administration on brain FAAH activity are well documented (Fegley *et al.* 2005; Gobbi *et al.* 2005; Kathuria *et al.* 2003). In the present study, chronic treatment with URB597 (.3 mg·kg^{-1}, once daily for 5 weeks) almost abolished FAAH activity in three representative regions of the rat brain (Figure 5)–midbrain [$F(1,24) = 6.12$, $p < .0001$], striatum [$F(1,27) = 7.30$, $p < .0001$], and hippocampus [$F(3,26) = 28.09$, $p < .0001$]–irrespective of whether the animals had or had not been exposed to CMS. Chronic mild stress itself had no significant effect on brain FAAH activity (Figure 5).

Effects of CMS on Brain Endocannabinoid Levels

Chronic mild stress did not significantly change anandamide levels in any of the five brain regions examined (Figure 6) [midbrain: $F(1,63) = 3.76$, ns; prefrontal cortex: $F(1,43) = .05$, ns; hippocampus: $F(1,63) = 2.17$, ns; striatum: $F(1,59) = 1.726$, ns; thalamus: $F(1,66) = 1.13$, ns]. In addition, CMS produced a small increase in 2-AG levels in the thalamus but had no effect in other brain regions (Table 1).
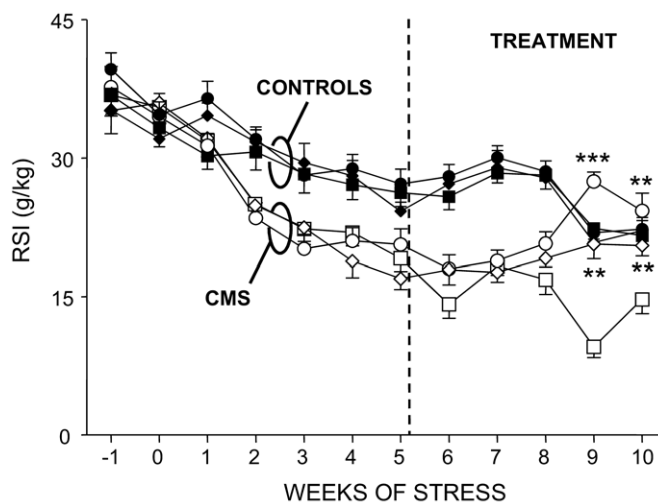
Figure 4. Effects of vehicle, URB597 (.3 mg·kg^{-1}, IP), and imipramine on RSI (20 mg·kg^{-1}, IP) in CMS-exposed and control rats. Values are expressed as mean ± SEM. Filled symbols, control rats; empty symbols, CMS-exposed rats; squares, vehicle; diamonds, URB597; circles, imipramine. *$p < .05$ versus CMS-exposed, vehicle-treated rats; **$p < .01$ versus control, vehicle-treated rats. IP, intraperitoneal; RSI, relative sucrose intake; CMS, chronic mild stress.

Figure 5. Effects of URB597 (.3 mg·kg^{-1}, IP) on FAAH activity in **(A)** midbrain, **(B)** striatum, and **(C)** hippocampus of CMS-exposed and control rats. Values are expressed as mean ± SEM. CMS, CMS-exposed rats; Veh, vehicle; URB, URB597. ***$p < .001$ versus vehicle. IP, intraperitoneal; FAAH, fatty acid amide hydrolase; CMS, chronic mild stress.

Effects of URB597 and Imipramine on Brain Endocannabinoid Levels

Consistent with its inhibitory effects on FAAH activity (Figure 5), chronic URB597 (.3 mg·kg^{-1}) increased anandamide content in the midbrain (Figure 6A) [$F(3,63) = 23.99$, $p < .0001$], thalamus (Figure 6B) [$F(3,66) = 1.98$, $p < .0001$], and striatum (Figure 6C) [$F(3,59) = 13.00$, $p < .0001$]. Surprisingly, the drug did not significantly affect anandamide levels in the hippocampus (Figure 6D) [$F(3,63) = .63$, ns] or prefrontal cortex [$F(3,43) = .13$, ns] (Figure 6E). These effects were comparable in CMS-exposed and control rats [interaction stress × treatment: midbrain: $F(3,63) = .29$, ns; striatum: $F(3,59) = .42$, ns; thalamus: $F(3,66) = .36$, ns; hippocampus: $F(3,63) = .03$, ns; prefrontal cortex: $F(3,43) = 1.973$, ns]. Moreover, URB597 dose-dependently elevated OEA and PEA content in the midbrain (Table 2) and other brain regions (data not shown) of control and CMS-exposed rats [interaction stress × treatment: OEA: $F(3,61) = .17$, ns; PEA: $F(3,61) = .47$, ns]. Finally, URB597 did not alter 2-AG levels in the midbrain (Table 1) [$F(3,59) = .87$, ns] or other brain regions [striatum: $F(3,62) = .89$, ns; thalamus: $F(3,68) = .21$, ns; hippocampus: $F(3,59) = 1.59$, ns; prefrontal cortex: $F(3,60) = 1.15$, ns]. Imipramine (20 mg·kg^{-1}) had no effect on anandamide levels in the brain of either control or CMS-exposed rats [interaction stress × treatment: $F(1,29) = .91$, ns].

Effects of CMS on CB1 Receptor mRNA Levels

Table 3 illustrates the effects of CMS and URB597 on CB1 mRNA levels in various regions of the rat brain. The CMS-exposed animals displayed an increase in CB1 mRNA in the prefrontal cortex [$F(3,49) = 14.40$, $p < .01$] and a decrease in CB1 mRNA in the midbrain [$F(2,56) = 4.66$, $p < .05$]. No such change was observed in the hippocampus [$F(3,42) = 1.44$, ns]. Treatment with URB597 (.3 mg · kg^{-1}) reversed the effects of CMS [prefrontal cortex: $F(3,53) = 7.21$, $p < .05$; midbrain: $F(2,56) = 4.38$, $p < .05$] but did not alter CB1 mRNA levels in control rats.

Discussion

The main finding of the present study is that chronic treatment with the potent and selective FAAH inhibitor URB597 normalizes BW gain and sucrose intake in rats exposed to CMS, a highly specific behavioral model of depression. These findings confirm and extend previous results, showing that URB597 enhances acute stress-coping responses in the mouse tail suspension test and the rat forced swim test (Gobbi *et al.* 2005; Kathuria *et al.* 2003; Patel *et al.* 2004; Patel and Hillard 2006).

Chronic mild stress was first formalized as an animal model of depression in 1987 (Willner *et al.* 1987) and, despite some initial criticisms (Forbes *et al.* 1996), has been since validated by numerous independent studies (Willner 2005). This paradigm offers important advantages over other models of depression: first, it induces in rodents an array of abnormal behaviors that are isomorphic with key features of human depression (Willner 2005); second, CMS-exposed rats–like depressed patients–respond to chronic, but not acute, treatment with antidepressant drugs (Willner 1997); third, psychostimulant agents, which can give false positives in acute stress-based models (De Pablo *et al.* 1989; Duncan *et al.* 1985; Steru *et al.* 1985), have no such effects in CMS (Papp *et al.* 1996).
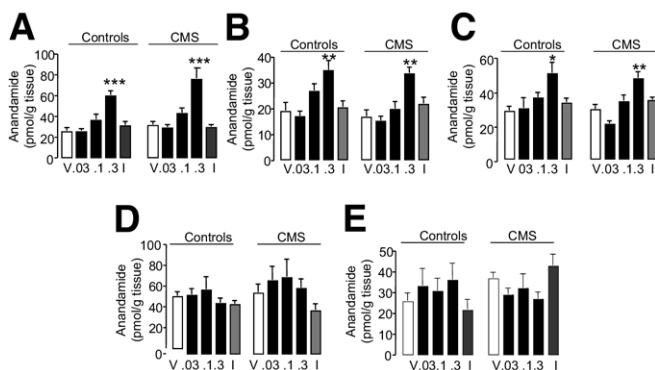

Figure 6. Effects of URB597 and imipramine on anandamide levels in **(A)** midbrain, **(B)** thalamus, **(C)** striatum, **(D)** hippocampus, and **(E)** prefrontal cortex in CMS-exposed and control rats. Values are expressed as mean ± SEM. V, vehicle; I, imipramine. URB597 doses are in mg·kg^{-1}. *$p < .05$ versus vehicle; **$p < .01$ versus vehicle; ***$p < .001$ versus vehicle. CMS, chronic mild stress.

Table 1. Effects of CMS on 2-AG Levels in Various Brain Regions (pmol/g Tissue)

	Control Subjects	CMS
Prefrontal cortex	5,372 ± 300	5,961 ± 383
Hippocampus	8,323 ± 450	8,142 ± 341
Striatum	8,758 ± 376	8,189 ± 268
Thalamus	11,474 ± 517	12,191 ± 461[a]
Midbrain	24,430 ± 1,043	26,356 ± 1,318

Values are expressed as mean ± SEM.

CMS produced a small increase in 2-AG levels in the thalamus [$F(1,68) = 4.07$, $p < .05$] but not in other brain regions [prefrontal cortex: $F(1,60) = 1.22$, ns; hippocampus: $F(1,59) = .05$, ns; amygdala: $F(1,65) = .54$, ns; striatum: $F(1,62) = 2.40$, ns; midbrain: $F(1,59) = .74$, ns].

CMS, chronic mild stress; 2-AG, 2-arachidonoylglycerol; ns, nonsignificant.
[a] $p < .05$ versus control subjects.

Table 2. Effects of URB597 on OEA and PEA Levels in the Midbrain (pmol/g Tissue)

	Vehicle	URB597 (.03 mg · kg^{-1})	URB597 (.1 mg · kg^{-1})	URB597 (.3 mg · kg^{-1})
OEA	491 ± 58	893 ± 245	1,686 ± 202[a]	2,079 ± 113[a]
PEA	750 ± 32	1,391 ± 139	3,202 ± 265[a]	3,839 ± 254[a]

Values are expressed as mean ± SEM. URB597 dose dependently elevated both OEA [$F(3,61) = 19.36, p < .0001$] and PEA [$F(3,61) = 47.95, p < .0001$] in this region.

OEA, oleoylethanolamide; PEA, palmitoylethanolamide.

[a]$p < .001$ versus vehicle.

The present results show that BW gain and sucrose intake are significantly reduced by CMS and are corrected by chronic treatment with URB597. Changes in sucrose intake are thought to reflect variations in operant appetitive behaviors and, therefore, to be a reliable index of anhedonic-like responses (Moreau 1997; Willner 1997). The alternative possibility that variations in sucrose intake might be secondary to BW changes (Forbes *et al.* 1996; Matthews *et al.* 1995) was ruled out by the lack of statistical correlation between CMS-induced variations in BW and ASI. Moreover, in agreement with previous findings (Willner 1997, 2005), CMS markedly affected RSI, a composite measure of intake normalized to BW. URB597 selectively normalized BW gain, ASI, and RSI in CMS-exposed but not control rats, suggesting that the FAAH inhibitor specifically targets neural processes altered by stress without affecting normal appetitive responses. Consistent with this idea, prior reports have shown that URB597 is devoid of orexigenic or rewarding properties (Fegley *et al.* 2005; Gobbi *et al.* 2005).

Previous research has shown that the CB1 antagonist rimonabant prevents the mood-enhancing actions of URB597, suggesting that such effects are based on the drug's ability to magnify endogenous anandamide signaling at CB1 receptors (Gobbi *et al.* 2005; Kathuria *et al.* 2003). In the present study, we could not provide a similar demonstration because of the intrinsic effects of rimonabant on BW, appetite, and hedonic drive to food (Arnone *et al.* 1997; Colombo *et al.* 1998), as well as on other behavioral end points affected by CMS (Griebel *et al.* 2005). Nevertheless, the idea that anandamide may be responsible for the effects of URB597 in CMS is supported by two sets of results. Firstly, direct cannabinoid agonists produce antidepressant-like responses in both CMS (Onaivi *et al.* 2006) and forced swim tests (Jiang *et al.* 2005). Secondly, as documented in the present report, chronic treatment with URB597 elevates anandamide levels in brain regions, such as midbrain and striatum, which are thought to participate in the induction of CMS-related behaviors (Bekris *et al.* 2005; Dziedzicka-Wasylewska *et al.* 1997; Papp *et al.* 1994).

It is also important to point out that the chronic URB597 regimen used in the present study did not increase anandamide levels in hippocampus and prefrontal cortex, even though it fully inhibited FAAH activity throughout the brain. This lack of effect, at variance with that of acutely administered URB597 (Fegley *et al.* 2005; Gobbi *et al.* 2005; Kathuria *et al.* 2003), suggests that prolonged exposure to the FAAH inhibitor may result in a down-regulation of anandamide mobilization in certain brain regions (e.g., hippocampus) but not others (e.g., midbrain). The mechanism underlying this hypothetical down-regulation of anandamide signaling remains undetermined.

Since acute or subchronic treatment with URB597 enhances firing of serotonergic and noradrenergic neurons in the midbrain (Gobbi *et al.* 2005), it is possible that persistent anandamide accumulation in this region might contribute to the antidepressant-like effects of URB597 in CMS (Haidkind *et al.* 2003; Liu *et al.* 2003; Papp *et al.* 1994). Testing this hypothesis will require, however, further experimentation.

In apparent contrast with our findings, subchronic treatment with rimonabant was found to improve hair coat appearance and reduce CMS-induced immobility in the forced swimming test in mice subjected to CMS (Griebel *et al.* 2005). We cannot provide an adequate explanation for this discrepancy, but two possibilities should be considered. Rimonabant may interact with an as yet uncharacterized cannabinoid-sensitive receptor distinct from CB1 or with a subpopulation of CB1 receptors distinct from those engaged by neurally released anandamide (Breivogel *et al.* 2001; Hajos *et al.* 2001; Haller *et al.* 2002; Rodgers *et al.* 2005). Alternatively, mice may differ from rats in their response to cannabinoid-acting drugs. In this context, it should be noted that rimonabant use in obese humans appears to be accompanied by increased, rather than reduced, anxiety and depression symptoms (Gelfand and Cannon 2006; Pi-Sunyer *et al.* 2006; Van Gaal *et al.* 2005).

We found that CMS is associated with an increase in CB1 receptor mRNA expression in the prefrontal cortex. This result is consistent with recent data showing an elevated CB1 receptor density in the prefrontal cortex of rats exposed to 21 days of chronic unpredictable stress (Hillard *et al.*, unpublished data, 2006). Such alteration might be related to multiple neurochemical changes described in CMS-exposed rats, including increased dopaminergic activity and decreased serotonergic activity (Bekris *et al.* 2005). Conversely, our finding that CB1 mRNA is reduced in the midbrain of CMS-exposed rats might parallel other neurotransmitter abnormalities observed in these animals, such as decreased D_2 receptor signaling in the substantia nigra and ventral tegmental area (Dziedzicka-Wasylewska *et al.* 1997) or desensitization of 5-hydroxy-tryptamine 1A (5-HT$_{1A}$) autoreceptors in the dorsal raphe nuclei (Froger *et al.* 2004).

Notably, our CMS protocol did not reproduce the changes in hippocampal CB1 protein and endocannabinoid levels previously reported by Hill *et al.* (2005). These authors showed that 2-AG and CB1 receptor levels are reduced in the hippocampus of rats subjected to a 21-day chronic unpredictable stress protocol. Duration of exposure to stress might well account for the different outcomes obtained in the two experiments. Irrespective

Table 3. Effects of URB597 (.3 mg · kg^{-1}, IP) on CB1 mRNA Receptor Levels in the Midbrain of CMS-Exposed and Control Animals

	Vehicle	URB597
Prefrontal Cortex		
Control Animals	10.84 ± 1.00	15.44 ± .77
CMS	40.15 ± 6.75[b]	24.29 ± 2.99[c]
Hippocampus		
Control Animals	7.98 ± 1.00	6.11 ± .50
CMS	7.29 ± 1.23	5.61 ± .43
Midbrain		
Control Animals	2.52 ± .18	2.41 ± .15
CMS	1.93 ± .07[a]	2.49 ± .12[c]

Messenger RNA levels were measured by RT-PCR and normalized by using glyceraldehyde 3-phosphate dehydrogenase as an internal standard. Values are expressed in arbitrary units, as mean ± SEM.

IP, intraperitoneal; mRNA, messenger RNA; CMS, chronic mild stress; RT = PCR, reverse transcription polymerase chain reaction.

[a]$p < .05$ versus control animals.

[b]$p < .01$ versus control animals.

[c]$p < .05$ versus vehicle.

of these speculations, our results show that prolonged treatment with URB597 opposes the changes in CB1 mRNA expression induced by CMS, without affecting CB1 mRNA levels in control animals. A plausible interpretation of these findings is that persistent FAAH inhibition normalizes neuroplastic changes induced by CMS in rats.

In conclusion, our studies confirm the antidepressant-like properties of URB597, documenting the ability of this FAAH inhibitor to correct the alterations associated with CMS, a naturalistic model of depression with high face, construct, and predictive validity. While the mechanism underlying the antidepressant-like effects of URB597 is still unknown, our results highlight the critical role of anandamide in the regulation of affective states and point to FAAH as a promising new target for the therapy of depression and other stress-related disorders.

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