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Role in Anxiety Behavior of the Endocannabinoid System in the Prefrontal Cortex

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In the present study we explored with a multidisciplinary approach, the role of anandamide (AEA) in the modulation of anxiety behavior at the level of the prefrontal cortex (PFC). Low doses of the metabolically stable AEA analog, methanandamide, microinjected into the PFC, produced an anxiolytic-like response in rats, whereas higher doses induced anxiety-like behaviors. Pretreatment with the selective antagonist of CB1 or TRPV1 receptors (AM251 and capsazepine, respectively) suggested that the anxiolytic effect evoked by AEA might be due to the interaction with the CB1 cannabinoid receptor, whereas vanilloid receptors seem to be involved in AEA anxiogenic action. When AEA contents in the PFC were increased by microinjecting the selective inhibitor of fatty acid amide hydrolase (FAAH), URB597, we observed an anxiolytic response only at low doses of the compound and no effect or even an anxiogenic profile at higher doses. In line with this, a marked decrease of AEA levels in the PFC, achieved by lentivirus-mediated local overexpression of FAAH, produced an anxiogenic response. These findings support an anxiolytic role for physiological increases in AEA in the PFC, whereas more marked increases or decreases of this endocannabinoid might lead to an anxiogenic response due to TRPV1 stimulation or the lack of CB1 activation, respectively.

Keywords: anandamide, anxiety, CB1 receptors, FAAH, prefrontal cortex, TRPV1 receptors

Introduction

Increasing evidence that low doses of cannabinoid agonists reduce anxiety-like behaviors in mice and rats is being reported (Patel and Hillard 2006; Rubino et al. 2007), thus suggesting an anxiolytic role for the endogenous cannabinoid signaling. In line with this hypothesis, pharmacological agents that enhance the endogenous cannabinoid signaling exert anxiolytic-like actions in rodents (Kathuria et al. 2003; Bortolato et al. 2006). Kathuria et al. showed that selective inhibitors of fatty acid amide hydrolase (FAAH), the enzyme responsible for the degradation of the endocannabinoid anandamide (AEA), while enhancing AEA levels in the brain, also reduced anxiety in the "elevated zero maze" and "isolation-induced ultrasonic vocalization" tests in rats in a CB1-dependent manner. Bortolato et al. (2006) extended these findings to the AEA transport inhibitor AM404. In rats tested by 3 different models—the elevated plus-maze, defensive withdrawal, and separation-induced ultrasonic vocalization—intraperitoneal injection of AM404 exerted a dose-dependent anxiolytic effect, which was prevented by the CB1 antagonist rimonabant. Similar results were obtained by Patel and Hillard (2006) in mice using the elevated plus-maze test. These data suggest that endogenously produced endocannabinoids are involved in the regulation of anxiety, likely via activation of brain CB1 receptors.

Different brain areas seem to be involved in the modulation of anxious states: among them, the prefrontal cortex (PFC) appears to have a key role in the modulation of anxiety behavior both in human and animal models. Increased neural activity was found in the orbitofrontal cortex and the anterior and posterior cingulate in subjects suffering from panic disorder (Bystritsky et al. 2001) and specific phobias (Dilger et al. 2003). Decreased binding of inhibitory γ-aminobutyric acid A (GABAA) receptors in the PFC was detected in panic and posttraumatic stress disorders (Malizia et al. 1998; Bremner et al. 2000). Recent imaging studies have revealed reduced 5-HT1AR binding in the anterior and posterior cingulate in patients with panic disorder (Neumeister et al. 2004). Increased neuronal activity in the PFC was also evident in animal models of anxiety. Stress- and anxiety-inducing stimuli consistently activate the PFC in rats (Singewald et al. 2003; Rubino et al. 2007), and lesioning or pharmacological inactivation of PFC by local administration of the benzodiazepine midazolam produces anxiolytic-like effects (Shah and Treit 2003, 2004). Moreover, it was reported by Laviolette and Grace (2006) that the cannabinoid signaling in the PFC can modulate the magnitude of neuronal emotional learning plasticity and memory formation through functional inputs from the basolateral amygdala. Recently, we have demonstrated that the stimulation of CB1 receptors in the PFC, amygdala, and hippocampus, with the subsequent activation of different signaling pathways, might represent the initial event underlying the anxiolytic effect of low doses of intraperitoneal Δ^9-tetrahydrocannabinol (THC) (Rubino et al. 2007).

In the present study, we investigated the possible existence of an endocannabinoid tone modulating anxiety behaviors in the PFC. To this aim, we applied a variety of complementary techniques including local endocannabinoid (AEA and 2-arachidonoyl glycerol [2-AG]) microinjection, FAAH pharmacological blocking, and reduction of the endocannabinoid tone by enhancing FAAH expression through lentivirus-mediated in vivo gene transfer, a system with high transduction efficiency and long-term expression in the central nervous system (Naldini and Verma 2000).

Materials and Methods

Animals

Adult Sprague-Dawley male rats (Charles River, Calco, Italy) weighing 150–175 g at the time of arrival were used in these experiments. Rats were grouped 3–4 per cage till surgery and then individually housed on

a 12:12 light/dark cycle with food/water available ad libitum. After surgery, rats were handled daily. Experiments were carried out in strict accordance with the guidelines released by the Italian Ministry of Health (D.L. 116/92 and D.L. 111/94-B) and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Drugs

Methanandamide (mAEA) was obtained from RBI (Natick, MA) and dissolved in ethanol, cremophor, and saline (1:1:18). URB597 (Alexis Biochemicals, San Diego, CA) and capsazepine (Sigma Aldrich, Milano, Italy) were dissolved in 20% dimethyl sulfoxide (DMSO). AM251, capsaicin (CPS), and 2-AG were obtained from Tocris Biosciences (Avonmounth, UK) and dissolved in Tween80, DMSO, and H_2Od (1:2:7) except for CPS that was dissolved in 20% DMSO.

Surgery

After 1 week from arrival, rats underwent surgery. Rats were anesthetized with chloral hydrate (400 mg/kg intraperitoneal) and a stainless steel guide cannula was stereotaxically implanted unilaterally in the PFC by applying coordinates from the Atlas of Paxinos and Watson (2005) (anterior-posterior 2.7 mm from bregma, lateral-medial 0.8 mm, dorsal-ventral 3 mm below the dura). The cannula was anchored with dental cement to a stainless steel screw in the skull. Following surgery, rats were given an injection of amoxicilline (20 mg/kg) and then allowed 7 days to recover before elevated plus-maze test.

In Vivo Delivery of Lentiviral Vectors

Three microliters of viral suspension, containing 4×10^6 lentiviral particles, were bilaterally injected into the PFC. Injections were made with a Hamilton syringe at a rate of 0.4 μl/min (1.5 μl at DV 3 mm and 1.5 μl at DV 2 mm per hemisphere) using a motorized injector. Following surgery, rats were given an injection of amoxicilline (20 mg/kg) and then allowed 7 days to recover before the elevated plus-maze test.

Elevated Plus-Maze

The elevated plus-maze comprised 2 open arms (50 cm × 10 cm) and 2 enclosed arms (50 cm × 10 cm × 40 cm) that extended from a common central platform (10 cm × 10 cm). The apparatus, constructed from gray iron, was elevated 50 cm above floor level. Testing was conducted in a quiet room under dim light (about 30 lux) during the early light phase (09.30–13.30 h) of the light cycle. At 7 days following surgery, rats were brought into the dimly lit behavioral testing room and left undisturbed for at least 1 h prior to testing. For all sessions, rats were individually placed on the central platform facing an open arm and allowed to freely explore the maze for 5 min. Between subjects, the maze was thoroughly cleaned with acetic acid 0.1%. Animals were tested 15 min after microinjected of mAEA or 1 h after microinjection of URB597. Behavioral parameters comprised both conventional spatiotemporal and ethological measures (Rodgers et al. 2003, 2005). Conventional measures were the frequencies of total, open, and closed arm entries (arm entry = all 4 paws into an arm) and the time spent in open, closed, and central parts of the maze. Ethological measures comprised frequency scores for head-dips (HD: exploratory movement of head/shoulders over the side of the maze), stretched attend postures (SAP: exploratory posture in which the body is stretched forward then retracted to the original position without any forward locomotion) and closed arm returns (CAR: exiting closed arm with forepaws only and doubling back into the same arm).

Construction of Lentiviral Vectors

We have generated lentiviral particles by using a "second generation" transient expression system. The system includes the pCMVDR8.74 packaging construct, which directs the expression of viral structural, enzymatic and regulatory proteins (Gag-Pol, Tat and Rev), the pMD2.G envelope expression construct, harboring the vesicular stomatitis virus G glycoprotein (VSV-G), and the pWPT/GFP transfer vector. The latter contains the green fluorescent protein (GFP) cDNA under the control of an intronless human elongation factor 1-α "short" (EF1-α short) promoter. All constructs were kindly provided by Dr Didier Trono (School of Life Sciences, Swiss Institute of Technology, Lausanne, Switzerland; http://tronolab.epfl.ch/).

The transfer vector pWPT/FAAH/GFP was generated as previously described (Osti et al. 2006). Briefly, an Eco RI-Hind III fragment containing the simian virus 40 (SV40)-early promoter was subcloned in the corresponding sites of pBluescriptII SK(−) (Stratagene M-Medical, Italy). A 2472-bp EcoRI fragment containing the cDNA of rat FAAH (kindly provided by Dr Dale G. Deutsch, State University of New York at Stony Brook, NY) was inserted into the EcoRI site of pBluescriptII SK(−), upstream of the SV40-early promoter. A BamHI-BssHII fragment containing the FAAH cDNA and the SV40-early promoter was finally isolated from pBluescriptII SK(−) and ligated into the BamHI-MluI sites of the pWPT/GFP vector. Thus, in the resulting construct (pWPT/FAAH/GFP) the FAAH and GFP cDNAs were placed under the transcriptional control of the EF1-α-short and SV40-early promoters, respectively, as depicted below.

Producer and Target Cell Culture

The 293FT cell line (Invitrogen, Milan, Italy), derived from 293 primary embryonic human kidney cells, was used to produce replication-incompetent lentiviral particles. C6 rat glioma cells were used as target cells for in vitro infection experiments. Both lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate (complete Dulbecco's modified Eagle's medium) and were grown at 37 °C with 5% CO_2 in a humidified incubator.

Generation of Lentiviral Particles and Target Cell Infection

Lentiviral particles pseudotyped with the VSV-G were produced by cotransfecting the pCMVDR8.74, pMD2.G, and pWPT/FAAH/GFP vectors into 293FT cells with the calcium phosphate precipitation method, as previously described (Osti et al. 2006). Briefly, 5×10^6 293FT cells were transfected with 20 μg of total plasmid DNA (3.5 μg pMD2.G vector; 6.5 μg pCMVDR8.74 vector; 10 mg pWPT/FAAH/GFP vector). Control particles for mock-transduction experiments were prepared with a pWPT/GFP vector, in which the EF1-α short promoter had been replaced by a SV40-early promoter. The calcium-DNA precipitate was formed by diluting the plasmids to a final volume of 450 μl 0.1× time echo (TE) (1× TE: 10 mM Tris pH 8.0; 1 mM ethylenediaminetetraacetic acid [EDTA]) and 50 μl 2.5 M CaCl2 and by adding this solution to 500 ml of 2× 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–buffered saline (281 mM NaCl, 100 mM HEPES, 1,5 mM Na_2HPO_4 pH 7.12). Precipitates were added to the cultures for 16 h; lentiviral particle-containing medium was collected 48 h after transfection and filtered through 0.45-μm pore-size cellulose acetate filters. Transduction experiments were performed in medium containing 4 μg/ml polybrene. Viral titration was performed by flow cytometer counting GFP-expressing C6 cells 48 h after infection. For in vitro FAAH-overexpression experiments, 30% confluent C6 cells were infected for 4 h with 10 Multiplicity of Infection (M.O.I.) lentiviral vectors; the particle-containing medium was replaced with fresh medium and cells were incubated at 37 °C for 48 h. Cells were then harvested and processed for western blot analysis and FAAH activity measurements.

Western Blotting

Forty-eight hours after lentiviral infection, C6 cells were gently detached by trypsinization, counted with an hemocytometer, centrifuged at 1000 × *g* for 10 min at 4 °C, washed in phosphate-buffered saline (PBS) at 1000 × *g* for 10 min at 4 °C and resuspended in ice-cold lysis buffer (30 ml/10^6 cells; composition: 10 mM Tris pH 7.5, 1 mM EDTA, 1% NONIDET P-40, Sigma protease-inhibitor cocktail at indicated dilutions). Cell suspensions were sonicated for 10 s and centrifuged at 13 000 × *g* for 20 min at 4 °C. Alternatively, the PFC was homogenized in an appropriate volume of ice-cold Buffer A (10 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride, 1 mM EDTA, 1 mM ethyleneglycol-bis

(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, 2 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 0.5% Triton, 5 mg/mL aprotinin, and 5 mg/mL leupeptin) and centrifuged at 13000 × *g* at 4 °C for 3 min. Equivalent amounts of protein of each lysate were combined with equal volumes of Laemmli sample buffer, boiled for 5 min, run onto 11% polyacrilamide gels (200 mV), and blotted onto nitrocellulose membranes (200 mA). After 1 h incubation in blocking solution (5% Marvel dried skimmed milk in phosphate-buffered saline-Tween 0.1%), membranes were incubated overnight at 4 °C with FAAH-specific primary antibodies (Cayman Chemical Company, Ann Arbor, Michigan; 1:500 in blocking solution). After five 10-min washes in PBS-Tween, blots were incubated for 60 min with peroxidase-conjugated secondary antibodies (anti-rabbit ImmunoPure antibodies, Pierce) diluted 1:1000 in blocking solution. Detection was performed using an enhanced chemiluminescence reagent (Amersham Biosciences, Milan, Italy).

FAAH Activity

FAAH activity was measured at 37 °C for 4 min in 100 µl of assay buffer (NaCl 116 mM; KCl 5.4 mM; $CaCl_2$ 1.8 mM; HEPES pH 7 25 mM; NaH_2PO_4 1 mM; $MgSO_4$ 0.8 mM) containing fatty acid-free bovine serum albumin (0.1%), protein from tissue homogenates (10 µg), 0.5 µM AEA, and anandamide[ethanolamine-^{3}H] (10000 cpm, specific activity 60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO). The reactions were stopped with charcoal/HCl (1:4, 100 µl), and radioactivity was measured in the aqueous layers by liquid scintillation counting.

Immunofluorescence

Rats were sacrificed by decapitation following the behavioral test, whole brains were rapidly removed, immediately frozen in liquid nitrogen and stored at –80 °C until used. Brains were then sectioned coronally at 20 µm using a cryostat and coronal sections mounted onto Superfrost Plus slides (Menzel Glaser, Braunschweig, Germany) were stored at –80 °C until processed. Briefly, after fixation with 4% paraformaldehyde in PBS for 15 min, slides were washed 2 times with PBS for 10 min at room temperature and then permeabilized for 15 min in PBS containing 0.1% (v/v) TritonX-100. Slides were then rinsed again 2 times 10 min each with PBS and blocked in 3% bovine serum albumin at room temperature for 1 h. They were then incubated with the primary FAAH antibody (Cayman Chemical Company), diluted 1:500 in PBS containing 10% blocking solution, at 4 °C overnight. After 2 washes in PBS, section were incubated for 1 h at room temperature with the secondary antibody Alexa Fluor 488 donkey anti-sheep IgG (H + L) (dilution 1:2000 in PBS containing 10% blocking solution; Molecular Probes, Parsley, UK) and then with DAPI 2 µg/mL for 5 min, followed by the final washes with PBS. After processing, the slides were coverslipped with fluorescent mounting medium and left at room temperature until completely dry before fluorescent microscope examination. Images of FAAH-positive cells were captured using a Olympus DP50 camera attached to an Olympus BX51-P polarizing/light microscope. Viewfinder Lite 1.0.135 software was used to import images from the camera, and digital images were processed using Adobe Photoshop 5.0, removing imperfections caused by dust particles and adjusting brightness levels.

Lipid Extraction and Endocannabinoid/PEA Measurement

Procedure of Tissue Extraction

Tissues were homogenized in 5 volumes of chloroform/methanol/Tris HCl 50 mM (2:1:1) containing 100 pmol of d_8-AEA, d_4-PEA, and d_5-2-AG. Deuterated standards were synthesized from d_8 arachidonic acid and ethanolamine or glycerol, or from d_4-ethanolamine and palmitic acid, as described, respectively, in Devane et al. (1992) and Bisogno et al. (1997). Homogenates were centrifuged at 13 000 × *g* for 16 min (4 °C), the aqueous phase plus debris were collected and extracted again twice with 1 volume of chloroform. The organic phases from the 3 extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized samples were then stored frozen at –80 °C under nitrogen atmosphere until analyzed.

Analysis of Endocannabinoid Contents

Lyophilized extracts were resuspended in chloroform/methanol 99:1 by volumes. The solutions were then purified by open bed chromatography on silica as described in Bisogno et al. (1997). Fractions eluted with chloroform/methanol 9:1 by volume (containing AEA, 2-AG, and palmitoylethanolamide [PEA]) were collected, the excess solvent was evaporated with a rotating evaporator, and aliquots were analyzed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC-APCI-MS) carried out under conditions described previously (Marsicano et al. 2002) and allowing the separations of 2-AG, AEA, and PEA. Mass spectrometry (MS) detection was carried out in the selected ion monitoring mode using *m/z* values of 356 and 348 (molecular ions +1 for deuterated and undeuterated AEA), 384.35 and 379.35 (molecular ions +1 for deuterated and undeuterated 2-AG), and 304 and 300 (molecular ions +1 for deuterated and undeuterated PEA). The area ratios between signals of deuterated and undeuterated AEA and PEA varied linearly with varying amounts of undeuterated AEA (30 fmol–100 pmol). The same applied to the area ratios between signals of deuterated and undeuterated 2-AG in the 100 pmol–20 nmol interval. AEA, PEA, and 2-AG levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas. The amounts of endocannabinoids and PEA were expressed as picomoles/milligrams of lipid extracted.

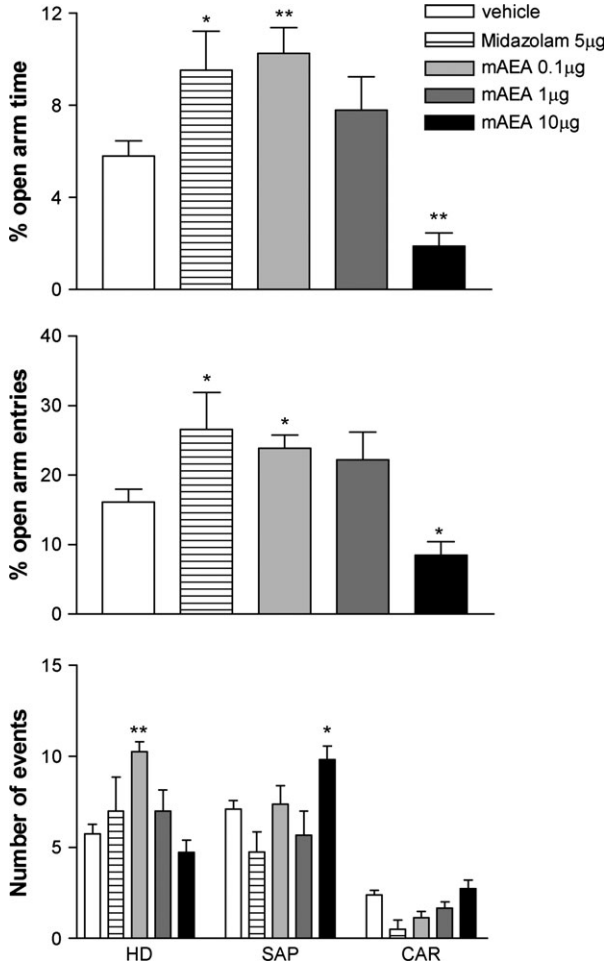

Figure 1. Effect of different doses of mAEA (0.1, 1, or 10 µg/rat) and midazolam (5 µg) administered into the PFC on rat anxiety behavior tested in the elevated plus-maze. The test was performed 15 min after the injection. The upper panel shows the percentage of time into the open arms, the middle panel shows the percentage of entries into the open arms, whereas the lower panel shows the number of ethological parameters observed. Bars represent the mean ± standard error of the mean. $^*P < 0.05$, $^{**}P < 0.01$ when compared with vehicles (1-way analysis of variance followed by Dunnet's test).

Statistical Analysis

The data presented in the figures are the mean ± standard error of the mean of at least 5 animals. Statistical analysis of the data was carried out by using 1-way analysis of variance followed by Dunnet or Tukey test performed by Prism (GraphPad Software, Inc., San Diego, CA).

Results

Figure 1 shows the effect of mAEA microinjection in the PFC on rat anxiety behavior tested in the elevated plus-maze.

The lower dose of mAEA (0.1 μg) significantly increased the percentage of time ($F_{3,28}$ = 14.57, $P < 0.0001$; $P < 0.01$ for post hoc comparison) and entries ($F_{3,28}$ = 11.38, $P < 0.0001$; $P < 0.05$ for post hoc comparison) onto the open arm, showing an anxiolytic effect. This effect was confirmed by the analysis of ethological parameters; in fact, a significant increase in HD ($F_{3,28}$ = 14.37, $P < 0.0001$; $P < 0.01$ for post hoc comparison) and a trend to decrease in CAR were observed.

The anxiolytic effect of 0.1 mg mAEA was comparable in intensity to that of the reference compound, midazolam, injected at the dose of 5 mg, as previously reported by Shah and Treit (2004).

At the dose of 1 μg, mAEA did not affect the anxiety behavior, whereas the highest tested dose (10 μg) significantly reduced the percentage of time ($F_{3,28}$ = 14.57, $P < 0.0001$; $P < 0.01$ for post hoc comparison) and entries ($F_{3,28}$ = 11.38, $P < 0.0001$; $P < 0.05$ for post hoc comparison) onto the open arm and increased "stretched attended postures" (SAP; $F_{3,28}$ = 3.97, $P < 0.05$; $P < 0.05$ for post hoc comparison), indicative of an anxiogenic effect.

To test whether CB1 receptors were involved in mAEA modulation of anxiety behavior, we pretreated rats with the selective CB1 antagonist AM251 (Fig. 2). AM251 (1 μg) was able to reverse the anxiolytic effect of mAEA 0.1 μg (Fig. 2*A*), but at the dose of 10 μg, it did not affect the anxiogenic response to 10 μg mAEA (Fig. 2*B*). Because it was already shown that both AEA and mAEA may evoke transient receptor potential vanilloid type-1 (TRPV1) channel-mediated responses at concentrations higher than those required to activate CB1 receptors (Ralevic et al. 2000; see Di Marzo et al. 2002 and Ross 2003 for reviews), we pretreated rats with the TRPV1 antagonist capsazepine to assess whether the anxiogenic effect of 10 μg mAEA could be due to the activation of TRPV1 receptor. First of all, we checked the effect of capsazepine per se and we observed a significant anxiolytic effect of this compound at the dose of 10 μg, as demonstrated by higher percentage of open arm time

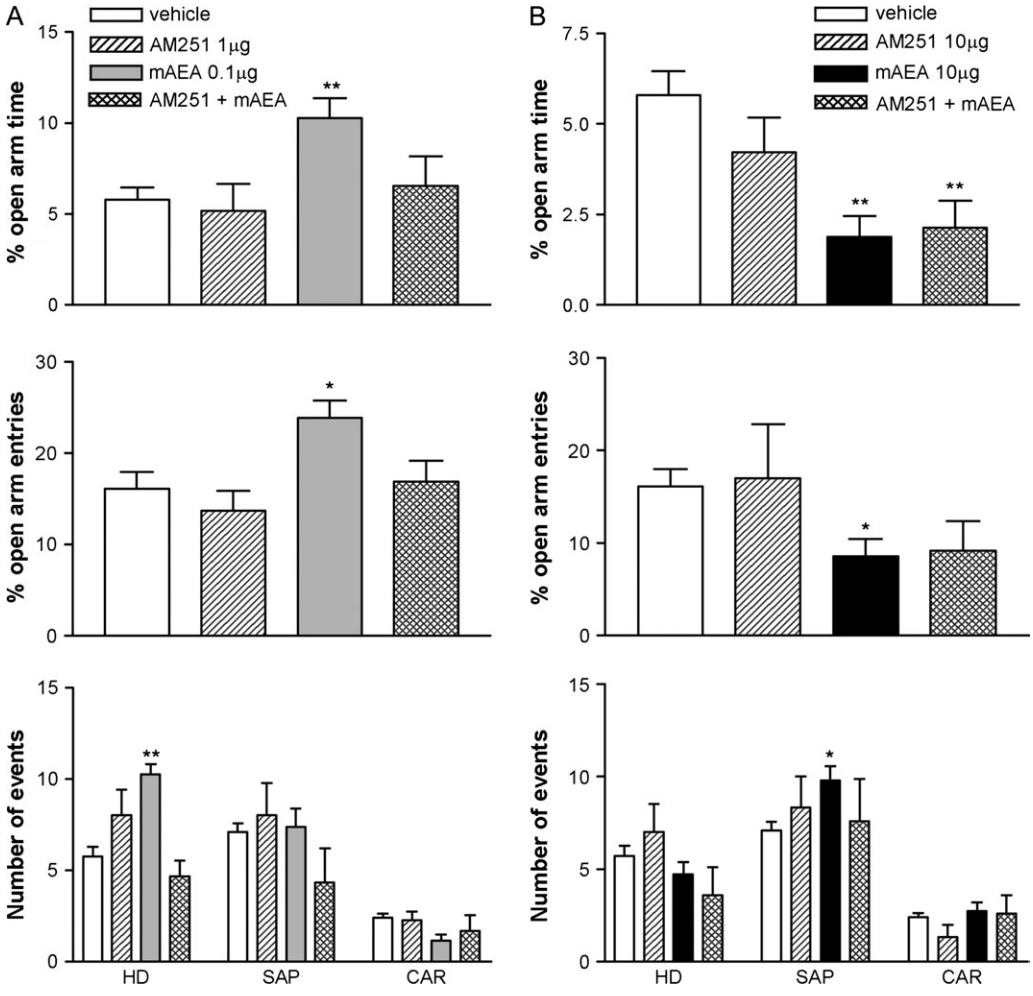

Figure 2. Effect of AM251 pretreatment ([*A*] 1 μg/rat or [*B*] 10 μg/rat, 5 min before methanadamide) on mAEA ([*A*] 0.1 μg/rat; [*B*] 10 μg/rat) modulation of rat anxiety behavior tested in the elevated plus-maze. The test was performed 15 min after mAEA injection. The upper panel shows the percentage of time into the open arms, the middle panel shows the percentage of entries into the open arms, whereas the lower panel shows the number of ethological parameters observed . Bars represent the mean ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with vehicles (1-way analysis of variance followed by Dunnet's test).

(capsazepine 13.00 ± 1.35 vs. vehicle 5.80 ± 0.70; $F_{2,9} = 8.41$, $P < 0.01$; $P < 0.01$ for post hoc comparison) and entries (capsazepine 29.17 ± 2.40 vs. vehicle 16.30 ± 1.97; $F_{2,9} = 13.77$, $P < 0.01$; $P < 0.01$ for post hoc comparison) in the elevated plus-maze. However, when administered at the dose of 5 μg, it was ineffective at altering anxiety behavior, so we used this dose to try to antagonize the effect of 10 μg mAEA. As reported in Figure 3, capsazepine prevented the decrease in the percentage of time and entries onto the open arm and counteracted as well the increased SAP produced by the highest dose of mAEA, demonstrating the involvement of TRPV1 receptors in the anxiogenic effect induced by high doses of mAEA. To further support a role for vanilloid receptors at inducing anxiogenesis, we injected increasing doses of CPS, the TRPV1 agonist, into the PFC and submitted rats to the EPM. As shown in Figure 4, CPS 1 μg was ineffective at altering anxiety behavior in rats; however, higher doses (5 and 10 μg) produced a clear anxiogenic response, as demonstrated by the significant decrease in percentage of time ($F_{3,10} = 3.968$, $P < 0.05$; $P < 0.05$ for post hoc comparison) and entries ($F_{3,10} = 10.85$, $P < 0.01$; $P < 0.05$ and $P < 0.01$ for post hoc comparison) onto the open arm. These data strengthen the hypothesis that activation of TRPV1 receptors might induce anxiety-like behaviors in rats.

Next, we investigated the impact of the modulation of the endocannabinoid tone in the PFC on anxiety behavior in rats. To this aim, we used URB597, a selective inhibitor of FAAH, to increase the endocannabinoid tone and, conversely, a lentivirus-mediated in vivo gene-transfer technique to overexpress FAAH in order to decrease the endocannabinoid tone.

Figure 5 shows the effect of PFC microinjections of different doses of URB597 on anxiety behavior in rats. URB597 produced a significant and dose-dependent block of FAAH activity within the PFC ($F_{3,40} = 18.5$, $P < 0.0001$; $P < 0.01$ for post hoc comparison) (Fig. 5*A*). The lower dose of URB597 (0.01 μg) induced a significant anxiolytic effect in rats, as demonstrated by the increased percentage of time ($F_{3,40} = 5.644$, $P < 0.01$; $P < 0.01$

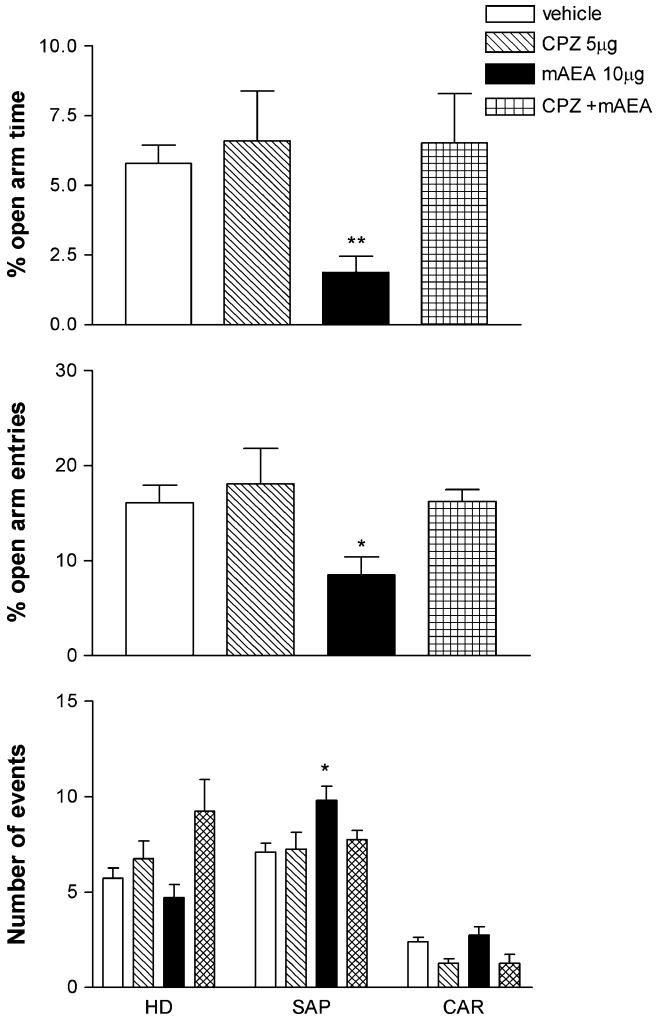

Figure 3. Effect of capsazepine pretreatment (CPZ; 5 μg/rat, 5 min before mAEA) on mAEA (10 μg/rat) modulation of rat anxiety behavior tested in the elevated plus-maze. The test was performed 15 min after mAEA injection. The upper panel shows the percentage of time into the open arms, the middle panel shows the percentage of entries into the open arms, whereas the lower panel shows the number of ethological parameters observed . Bars represent the mean ± standard error of the mean. $^*P < 0.05$, $^{**}P < 0.01$ when compared with vehicles (1-way analysis of variance followed by Dunnet's test).

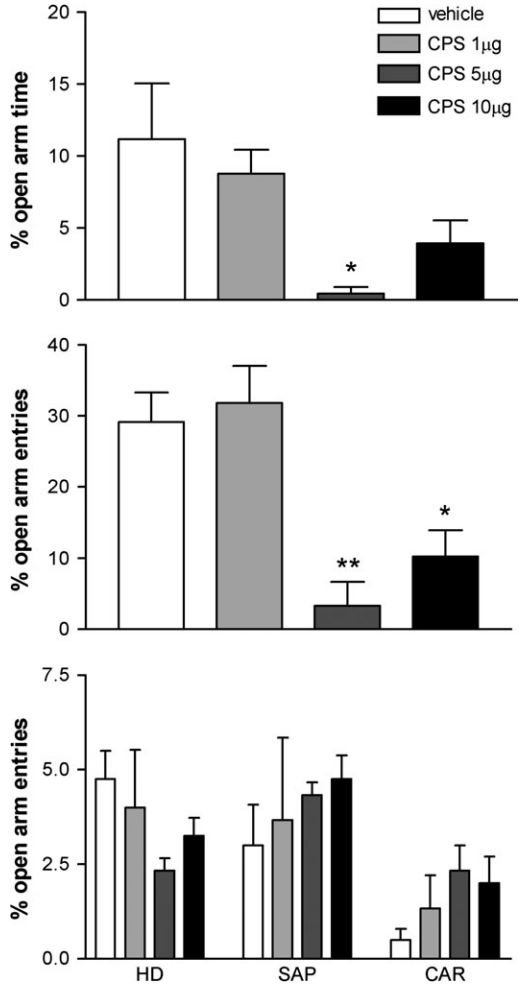

Figure 4. Effect of different doses of CPS (1, 5, or 10 μg/rat) administered into the PFC on rat anxiety behavior tested in the elevated plus-maze. The test was performed 15 min after the injection. The upper panel shows the percentage of time into the open arms, the middle panel shows the percentage of entries into the open arms, whereas the lower panel shows the number of ethological parameters observed. Bars represent the mean ± standard error of the mean. $^*P < 0.05$, $^{**}P < 0.01$ when compared with vehicles (1-way analysis of variance followed by Dunnet's test).

for post hoc comparison) and entries ($F_{3,40}$ = 5.409, $P < 0.01$; $P < 0.05$ for post hoc comparison) onto the open arm, as well as by the raise in HD ($F_{3,40}$ = 2.84, $P < 0.05$; $P < 0.05$ for post hoc comparison) and decrease in CAR ($F_{3,40}$ = 5.887, $P < 0.01$; $P < 0.01$ for post hoc comparison) (Fig. 5*B*). Higher doses of this selective FAAH inhibitor (0.1 and 1 μg) could no longer affect the anxiety response of treated animals or even show a trend towards an anxiogenic effect (URB597 1 μg), confirming that at high doses endocannabinoids switch to an anxiogenic profile.

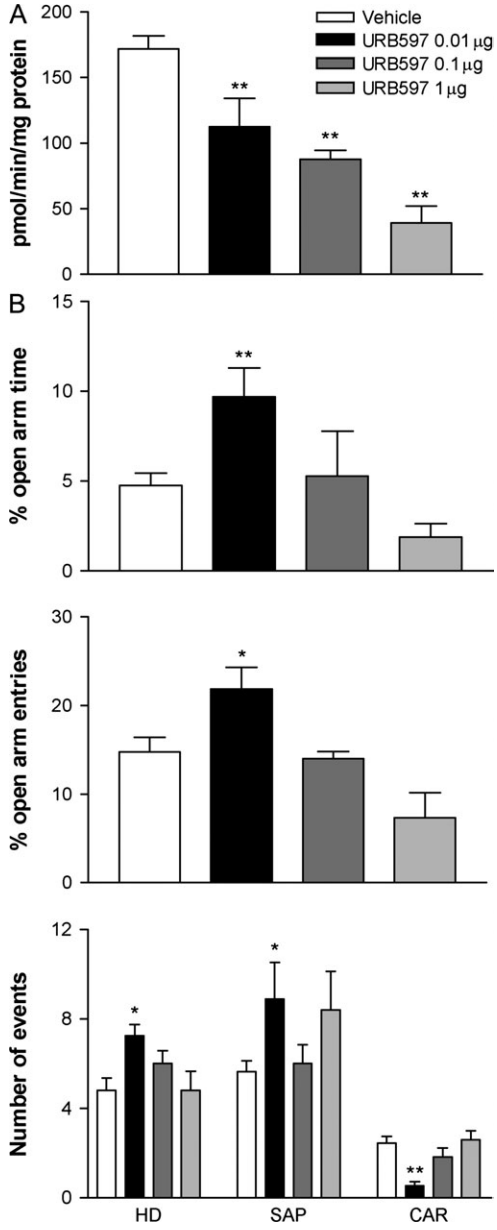

Figure 5. Effects of different doses of URB597 (0.01, 0.1, 1 μg/rat) administered into the PFC of rats on FAAH enzymatic activity (*A*) and anxiety behavior (*B*) tested 1 h after injection. In (*B*), the upper panel shows the percentage of time into the open arms, the middle panel shows the percentage of entries into the open arms, whereas the lower panel shows the number of ethological parameters observed. Bars represent the mean ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with vehicles (1-way analysis of variance followed by Dunnet's test).

Prior to lentiviral transduction experiments, FAAH overexpression and activity were tested in in vitro-infected C6 rat glioma cell lines. The transduction efficiency of lentiviral particles (Multiplicity Of Infection = 10) in C6 cells ranged between 85% and 90%. C6 cells transduced with lentiviral particles prepared with the pWPT/FAAH/GFP vector overexpressed FAAH by approximately 8-fold when compared with mock-transduced controls, expressing basal levels of the endogenous protein (Fig. 6). Consistently, FAAH-overexpressing cells displayed a 9-fold increase of FAAH enzymatic activity (Fig. 6).

The lentiviral vectors were then microinjected into the PFC of rats and the transduction efficiency was evaluated 7 days postinfection. Figure 7*A* shows an immunofluorescence experiment performed by probing rat coronal sections with a FAAH-specific polyclonal antibody. The fluorescent signal confirmed the overexpression of the FAAH protein in the microinjection site. We then used western blotting analysis to estimate FAAH overexpression: as shown in Figure 7*B* FAAH-transduced animals produced a 130% increase of protein levels versus mock-transduced ($F_{2,9}$ = 5.491, $P < 0.05$; $P < 0.05$ for post hoc comparison) or vehicle-injected rats ($F_{2,9}$ = 5.491, $P < 0.05$; $P < 0.05$ for post hoc comparison). In line with this result, the enzymatic activity of FAAH in the PFC of FAAH-transduced rats was about 90% higher than in vehicle-injected controls ($F_{2,26}$ = 16.54, $P < 0.0001$; $P < 0.001$ for post hoc comparison) (Fig. 7*C*). Unexpectedly, mock-transduced animals also showed

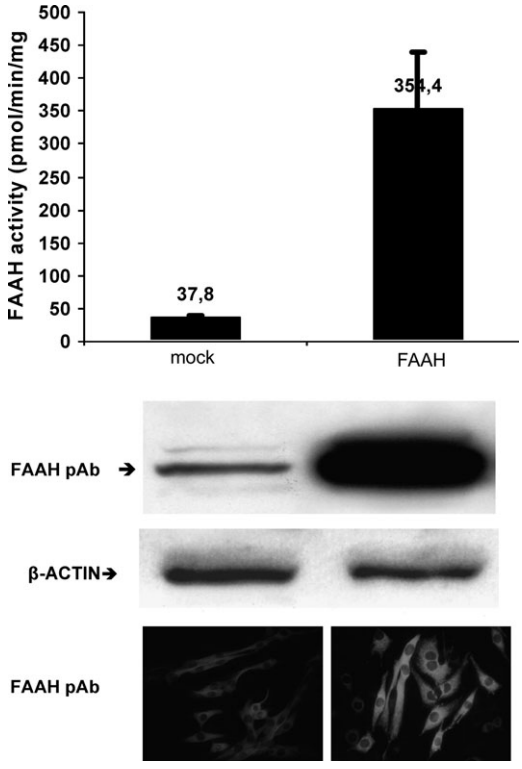

Figure 6. Expression and activity of FAAH in rat C6 glioma cells transduced with a lentiviral vector prepared with the pWPT/FAAH/GFP construct, containing the FAAH cDNA (FAAH), or mock infected with a vector prepared with the control pWPT/GFP construct (mock). Western blot analysis and immunofluorescence were performed using a FAAH-specific polyclonal antibody; loading control of blots was performed using β-actin antibodies. Enzymatic activity is expressed as pmol/min/mg protein. Experiments were repeated twice with identical results.

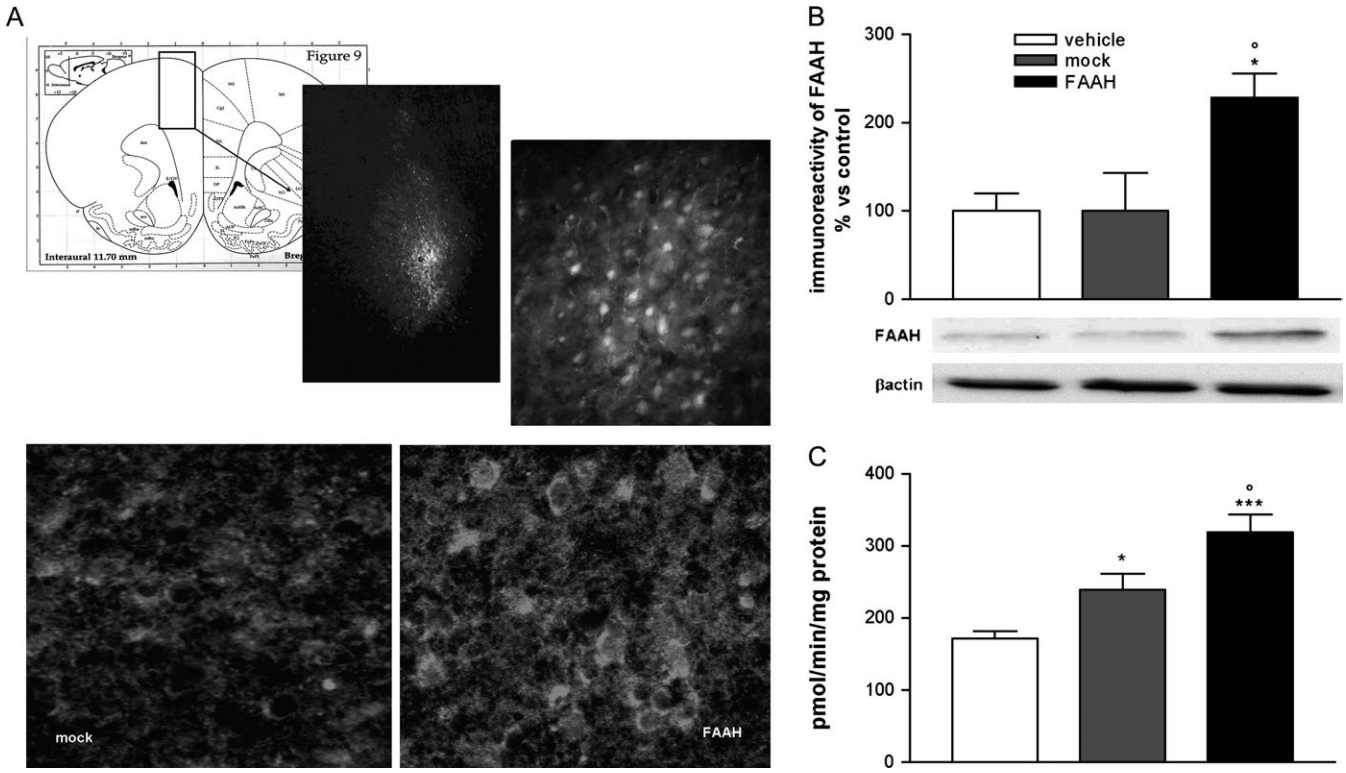

Figure 7. Expression (*A, B*) and activity (*C*) of FAAH in rat PFC transduced with a lentiviral vector containing the FAAH cDNA (FAAH) or mock infected (mock). Immunofluorescence (*A*) and western blot analysis (*B*) were performed using a FAAH-specific polyclonal antibody; loading control of blots was performed using β-actin antibodies. Enzymatic activity (*C*) is expressed as pmol/min/mg protein. In (*A*) immunofluorescence staining for GFP (upper panel) and FAAH (lower panel) of sections from the PFC. In the upper panel, camera lucida drawing of the brain section containing PFC from the Paxinos atlas and the area surrounding the injection site expressing GFP at different magnifications are shown.

increased FAAH activity when compared with controls (39%; $F_{2,26}$=16.54, $P < 0.0001$; $P < 0.05$ for post hoc comparison); however, this activity was significantly lower than that of FAAH-overexpressing rats ($F_{2,26}$=16.54, $P < 0.0001$; $P < 0.05$ for post hoc comparison). Moreover, local AEA content in the PFC of FAAH-transduced rats was significantly decreased when compared with vehicle-injected ones (Table 1), but, again, mock-transduced animals also exhibited decreased levels. A similar pattern was observed also for PEA, a member of the fatty acid ethanolamide family that is a substrate for FAAH in the brain (Table 1). Interestingly, the decrease of AEA was paralleled by increased 2-AG levels, which raised up to about 6-fold in mock-transduced rats, but up to about 8-fold in FAAH-transduced animals (Table 1).

On the basis of these results, we decided to microinject 2-AG in the PFC to assess the activity of this compound on anxiety behavior. As reported in Figure 8, at all tested doses (0.1–10 μg) 2-AG did not significantly modify the anxiety response in rats.

We then monitored anxiety behavior in FAAH-transduced rats. Decreased endocannabinoid tone in the PFC induced in rats an anxiogenic profile (Fig. 9). When tested in the EPM, FAAH-transduced rats presented decreased percentage of time ($F_{2,19}$=13.88, $P < 0.001$; $P < 0.001$ for post hoc comparison between FAAH-transduced and mock-transduced rats and $P < 0.01$ between FAAH-transduced and vehicle animals) and entries ($F_{2,19}$=7.55, $P < 0.01$; $P < 0.01$ for post hoc comparison between FAAH-transduced and mock-transduced rats and $P < 0.05$ between FAAH-transduced and vehicle animals) onto the open arms. This anxiogenic response was confirmed by ethological parameters; in fact, rats overexpressing FAAH showed decreased HD ($F_{2,19}$ = 7.66, $P < 0.01$; $P < 0.01$ for post hoc comparison between FAAH-transduced and mock rats and $P < 0.05$ between FAAH-transduced and vehicle animals) and increased CAR ($F_{2,19}$ = 3.795, $P < 0.05$; $P < 0.05$ for post hoc comparison between FAAH-transduced and mock-transduced rats). Besides the observed alterations in anxiety responses, transduced animals did not present significant changes in gross behavior (data not shown) or locomotor activity, as suggested by the number of closed arm entries (Fig. 9).

Table 1
Effect of lentiviral vector injection containing the FAAH cDNA on endocannabinoid contents in the PFC

	Vehicle	Mock	FAAH
AEA (pmol/mg lipid)	1.7 ± 0.35	0.87 ± 0.05	0.80 ± 0.06
PEA (pmol/mg lipid)	8.5 ± 1.7	3.4 ± 0.3	3.3 ± 0.2
2-AG (pmol/mg lipid)	49.75 ± 17.06	299.02 ± 35.65	385.35 ± 20.70

Discussion

The main result of this study is that AEA in the PFC plays a key role in modulating anxiety behavior. Specifically, low doses of the metabolically stable AEA analog, mAEA, produced an anxiolytic-like response in rats, whereas higher doses induced anxiety-like behavior. The biphasic effect of AEA, with low doses producing discernible and different effects from those of high doses, has already been described using a series of

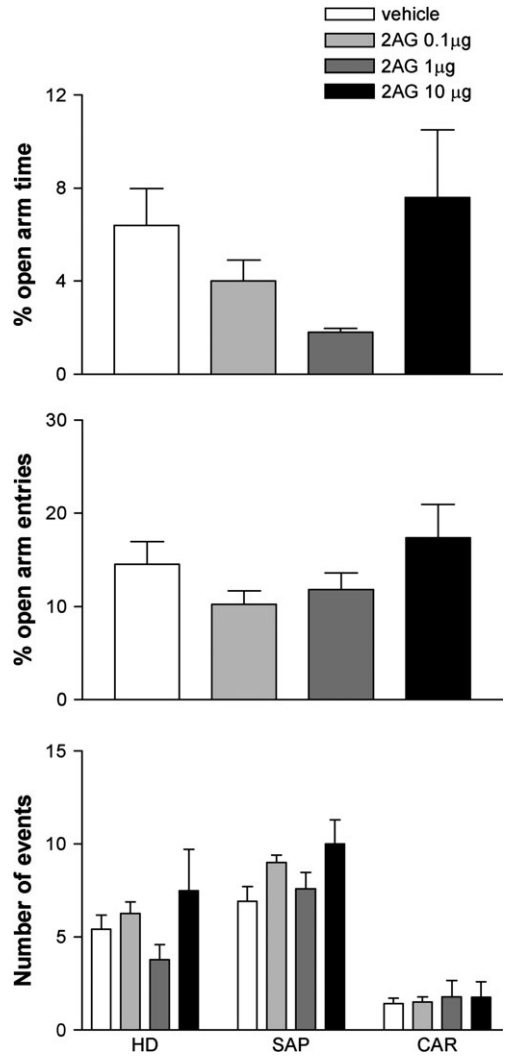

Figure 8. Effect of different doses of 2AG (0.1, 1, or 10 μg/rat) administered into the PFC on rat anxiety behavior tested in the elevated plus-maze. The test was performed 15 min after injection. The upper panel shows the percentage of time into the open arms, the middle panel shows the percentage of entries into the open arms, whereas the lower panel shows the number of ethological parameters observed. Bars represent the mean ± standard error of the mean.

physiological and behavioral assays (Sulcova et al. 1998). In particular, Sulcova et al. (1998) observed stimulated motor activity and defecation rate in the open field, increased motility on the ring and aggressive behavior in "timid" mice treated with 0.01 mg/kg AEA, and opposite effects of inhibitory nature with 100/1000-fold higher AEA concentrations. In the present study, the anxiolytic dose of mAEA was 100-fold lower than the anxiogenic one, the dose range required for the opposite effect resembling the one described by Sulcova et al. (1998). Different mechanisms—which were not tested nor further investigated—were suggested by these authors to explain the biphasic effect of AEA. Regarding the mAEA-induced modulation of anxiety behavior, we suggest that the opposite effects produced by low and high doses of this compound might be due to its ability to interact with different receptors. In our hands, the anxiolytic effect of low AEA was reversed by administration of the specific CB1 receptor antagonist AM251, whereas the anxiogenic effect was inhibited by pretreatment with capsazepine (5 μg), a TRPV1 receptor antagonist. This picture suggests that the anxiolytic effect evoked by AEA might be due to the interaction with the CB1 cannabinoid receptor, whereas vanilloid receptors seem to be involved in AEA anxiogenic action. The reason why AEA-induced anxiogenesis appears at high doses could lie in the fact that the intrinsic efficacy of AEA at TRPV1 is relatively low in comparison to that observed at the CB1 receptor (see Ross 2003). The anxiolytic effect observed with a higher dose of capsazepine (10 μg) and the anxiogenic-like response produced by CPS microinjection further support the hypothesis that vanilloid receptors could mediate the anxiogenic effect induced by AEA. The involvement of vanilloid receptors in anxiety behavior has been recently suggested by Kasckow et al. (2004), who provided preliminary evidences on the anxiogenic effect of vanilloid agonists such as olvanil and on the anxiolytic properties of capsazepine. The results obtained by Kaschow et al. as well as our findings point to the possibility of a therapeutic exploitation of vanilloid antagonists for treating anxiety. Accordingly, Marsch et al. reported that TRPV1 "null" mice exhibit a significantly reduced response to anxiogenic stimuli (Marsch et al. 2007).

When we increased AEA contents by administering the selective inhibitor of FAAH, URB597, we observed an anxiolytic effect, but, again, depending on the microinjected dose: URB597 was effective at decreasing anxiety behavior only at the lowest tested dose (0.01 μg) and lost its effect or even induced an anxiogenic profile at higher doses. The lowest dose of URB597 produced the lowest decrease in FAAH activity (34%) and, consistently, the smallest increase in AEA level. All these findings point toward an anxiolytic role of physiological increases in AEA levels at the synapses present in the PFC. In contrast, when endogenous levels of AEA are increased beyond a certain threshold, for example, by administration of high doses of URB597, TRPV1 receptors may become activated, thus leading to an overall null net effect on anxiety or even an anxiogenic response.

To further corroborate the findings described above, we tested the effect of pronounced decreases in PFC AEA contents on anxiety behavior in rats. To this aim, we used an innovative approach represented by lentiviral particles designed to transduce and overexpress the cDNA encoding for rat FAAH. In contrast to the use of FAAH *null* mice, this tool allows to overexpress the enzyme responsible for AEA degradation in a time- and site-selective way. The microinjection of lentiviral particles in the PFC of rats led to local FAAH overexpression paralleled by increased FAAH activity, by a significant loss of AEA and by an anxiogenic response. Mock-transduced rats did not show any alteration in anxiety behavior, no FAAH overexpression, but, surprisingly, increased FAAH activity and low AEA levels. However, this group showed a 39% increase in FAAH activity versus vehicle-injected controls, whereas in FAAH-transduced rats, the enzymatic activity raised by 86%, a value significantly higher. In a previous report, it was shown that the HIV-1 glycoprotein gp120 activates FAAH, and decreases endogenous levels of AEA, in the brain neocortex of rats (Maccarrone et al. 2004). However, the second-generation lentiviral particles used in our work are pseudotyped with the G glycoprotein of the vescicular stomatitis virus and do not express gp120 on their surface. It is possible, however, that other viral coat glycoproteins may induce FAAH activation in the rodent brain in a nonspecific fashion; this possibility deserves further investigation.

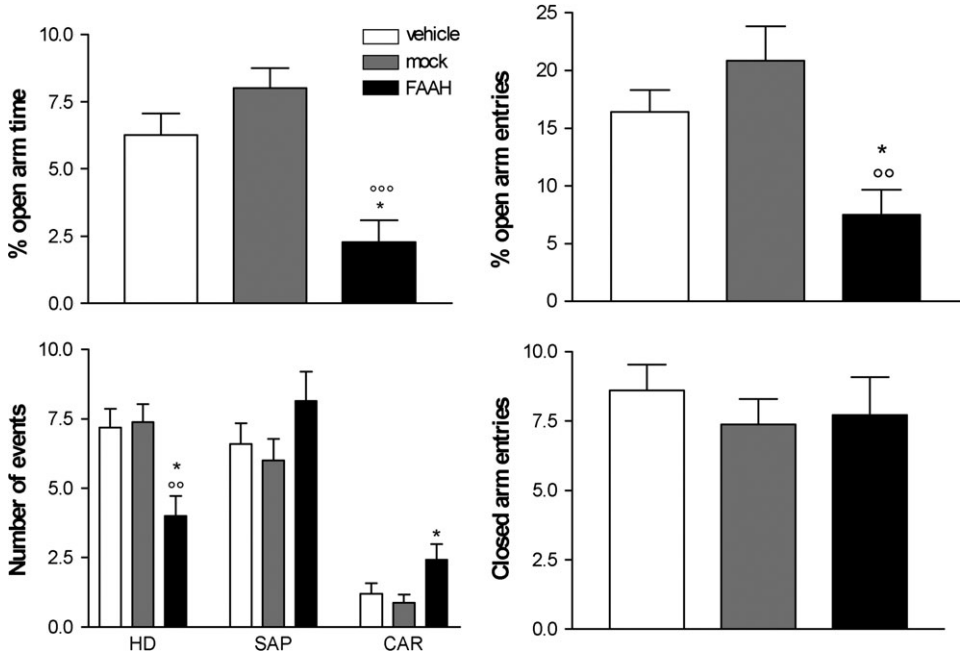

Figure 9. Effects of FAAH overexpression in the PFC of rats on anxiety behavior tested in the elevated plus-maze. The test was performed 7 days after lentiviral infusion. The upper panels show the percentage of time and entries into the open arms, whereas the lower panels show the number of ethological parameters observed and the number of entries into the enclosed arms. Bars represent the mean ± standard error of the mean. $^{*}P < 0.05$ when compared with vehicles. $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with mock rats (1-way analysis of variance followed by Tukey's test).

Regarding AEA contents, mock- and FAAH-transduced rats exhibited approximately the same amount of this endocannabinoid and this was quite surprising because a lower AEA content was expected in the FAAH-overexpressing group. A possible explanation to this finding is that lower AEA contents could not be measured due to a "floor" effect. It has been already reported elsewhere (Saghatelian et al. 2006) that amounts of AEA around 20 pmol/g tissue are "barely detectable," and in our experiments, AEA amounts in mock- and FAAH-transduced animals were about 40 pmol/g tissue. Moreover, in addition to its activity on AEA hydrolysis, FAAH has been reported to catabolize other bioactive lipids such as oleamide or *N*-acyl taurines (Saghatelian et al. 2006). Although the relevance of these compounds in modulating anxiety behavior is not known, we cannot rule out that they might contribute to the anxiogenic phenotype observed in FAAH-transduced rats. Alternatively, potential artifactual rises in AEA levels following brain dissection could have diluted the effect of FAAH overexpression. In fact, although care was taken here to rapidly dissect the PFC for endocannabinoid analysis, the possibility cannot be excluded that even a little postmortem time might have been sufficient to increase AEA levels, thus compensating for the further increase of FAAH activity in FAAH-transduced versus mock-transduced rats. As to the finding of increased 2-AG levels in mock- and, particularly, FAAH-transduced animals, this was surprising as previous reports have shown either no changes or increases following pharmacological FAAH "inactivation" in rats (Kathuria et al. 2003; De Lago et al. 2005; Maione et al. 2006). Apart from suggesting a direct effect of lentivirus injection per se on 2-AG metabolism, opposite to that induced on AEA metabolism (see above), we might suggest that the pharmacological blockade of FAAH by acute injection of FAAH inhibitors could produce effects that are different from those obtained with the stable overexpression of the enzyme, which might provoke adaptive mechanisms. From a behavioral point of view, however, the anxiogenic profile exhibited by rats transduced with FAAH lentiviral vectors in the PFC is likely to be due to the observed decreased levels of AEA. In fact, although this behavioral response could in part be due to the observed raise of 2-AG, whose levels in the PFC are several-fold higher than those of AEA, this compound, when administered alone, had little if any effect on anxiety at all doses tested, including those in the same range of the Δ for the observed increase of 2-AG levels in FAAH-transduced rats.

In conclusion, our experiments suggest that in the PFC, tonic activation of CB1 and, at higher concentrations, TRPV1 receptors by AEA, but not 2-AG, control anxiety in opposite ways. If, for any reason, the tissue levels of AEA become either too low or too high, thus leading to lack of CB1 activation or TRPV1 stimulation, respectively, anxiogenic responses are observed. This hypothesis, apart from being in agreement with the data reported here using several different experimental approaches and methodologies, explains why some authors (e.g., Naidu et al. 2007) have reported that FAAH inhibitors fail to produce anxiolytic responses in rodents. In view of our findings, one can postulate that the efficacy of such compounds might depend not only on the dose used but also on the past history of the animal (and hence its baseline endocannabinoid tone) or the type of stressor applied (which can produce different elevations of the endocannabinoid tone) to measure such behaviors. From a therapeutical point of view, it could therefore be of interest to test as antianxiety therapies compounds that can slightly increase the endocannabinoid tone alone or in combination with vanilloid antagonists.

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