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Reduced anxiety-like behaviour induced by genetic and pharmacological inhibition of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH) is mediated by CB1 receptors

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Abstract

Anandamide and 2-arachidonoyl glycerol, referred to as endocannabinoids (eCBs), are the endogenous agonists for the cannabinoid receptor type 1 (CB1). Several pieces of evidence support a role for eCBs in the attenuation of anxiety-related behaviours, although the precise mechanism has remained uncertain. The fatty acid amid hydrolase (FAAH), an enzyme responsible for the degradation of eCBs, has emerged as a promising target for anxiety-related disorders, since FAAH inhibitors are able to increase the levels of anandamide and thereby induce anxiolytic-like effects in rodents. The present study adopted both genetic and pharmacological approaches and tested the hypothesis that FAAH-deficient ($FAAH^{-/-}$) mice as well as C57BL/6N mice treated with an FAAH inhibitor (URB597) would express reduced anxiety-like responses. Furthermore, as it is known that anandamide can bind several other targets than CB1 receptors, we investigated whether FAAH inhibition reduces anxiety via CB1 receptors. $FAAH^{-/-}$ mice showed reduced anxiety both in the elevated plus maze and in the light-dark test. These genotype-related differences were prevented by the CB1 receptor antagonist rimonabant (3 mg/kg). Moreover, URB597 (1 mg/kg) induced an anxiolytic-like effect in C57BL/6N mice exposed to the elevated plus maze, which was prevented by rimonabant (3 mg/kg). The present work provides genetic and pharmacological evidence supporting the inhibition of FAAH as an important mechanism for the alleviation of anxiety. In addition, it indicates an increased activation of CB1 receptors as a mechanism underlying the effects of FAAH inhibition in two models of anxiety.

Keywords: Endocannabinoids; Anandamide; Fatty acid amide hydrolase; CB1 receptor; Anxiety; FAAH knock out mice

1. Introduction

The herb *Cannabis sativa* may induce a diversity of emotional responses ranging from anxiolytic and relaxing effects to the induction of panic attacks (Hall and Solowij, 1998). Divergences have also been observed in both humans and rodents after the administration of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main active compound of marijuana, or its synthetic counterparts (Berrendero and Maldonado, 2002; Marco et al., 2004; Patel and Hillard, 2006; Zuardi et al., 1982).

The mechanisms underlying these bidirectional effects remain to be determined. In the brain, cannabinoids activate the type 1 cannabinoid (CB1) receptor, which is densely expressed in a number of regions related to the modulation of fear and anxiety (Mackie, 2005; Pacher et al., 2006). However, several variables are likely to interfere with the activity of these compounds on experimental anxiety (for a review, see Viveros et al., 2005). First, the effect may depend on the dose administered. In general, low doses tend to be anxiolytic and high doses tend to be anxiogenic (Marco et al., 2004). Second, the characteristics of the experimental environment,

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such as the intensity of illumination, may influence the effects of CB1 activation or blockade (Haller et al., 2004a; Naidu et al., 2007). Third, the various protocols employed for measuring anxiety may generate diverse aversive states, with which cannabinoids may interfere in opposite ways (Viveros et al., 2005). Finally, the previous history of the subjects, such as exposure to stresses or drug treatments, may also be of relevance to the response to cannabinoids (Rodgers et al., 2005).

An alternative approach to the direct activation of CB1 receptors is the enhancement of the availability of the endogenous ligands, referred to as endocannabinoids (eCBs), of which anandamide and 2-arachidonoylglycerol (2-AG) are the mostly investigated (Piomelli, 2003). Their actions are terminated by a putative uptake process, followed by degradation by fatty acid amide hydrolase (FAAH) and by monoacylglycerol lipase (McKinney and Cravatt, 2005), respectively. Specific inhibitors of FAAH have been developed that significantly increase the brain levels of anandamide, but not 2-AG, thereby potentiating the effects of anandamide (Kathuria et al., 2003). FAAH inhibitors induce analgesia, enhance memory extinction and attenuate anxiety via an increased activation of CB1 receptors (Kathuria et al., 2003; Patel and Hillard, 2006; Varvel et al., 2007). However, anandamide is a promiscuous neuromodulator that may bind to other sites in the brain, implying that alternative mechanisms, apart from the activation of CB1 receptors, may be involved in its actions (Ross, 2003). For example, the transient receptor potential vanilloid type 1 channel (TRPV1) is activated by anandamide and located in several brain regions related to emotions (Cristino et al., 2006). TRPV1 was shown to have a role on the modulation of both anxiety and conditioned fear (Marsch et al., 2007). Therefore, increasing the endogenous levels of anandamide may induce effects that are not only CB1-mediated, but also dependent on other receptors.

Apart from the pharmacological studies mentioned above, genetic approaches have also proved to be very useful for the study of the endocannabinoid system. Cravatt et al. (2001) generated mouse mutants lacking the FAAH gene ($FAAH^{-/-}$ mice). These animals have a 10–15-fold increase in the levels of anandamide and an enhanced response to the injection of this endocannabinoid, altered nociceptive responses and enhanced memory extinction (Cravatt et al., 2001; Varvel et al., 2007). Despite these results, the response of $FAAH^{-/-}$ mice in models of anxiety has remained uncertain. Furthermore, behavioural changes not related to an increased activation of the CB1 receptor have also been observed after genetic inhibition of FAAH (Wise et al., 2007).

Therefore, the aim of this study was to test the notion that $FAAH^{-/-}$ mice might show a reduced anxiety-like behaviour as compared to their wild-type (WT) littermates. The elevated plus maze (EPM) and the light dark test (LDT) were employed as animal models. Furthermore, in order to mimic these experiments pharmacologically, studies were conducted with injections of an FAAH inhibitor in C57BL/6N mice. Finally, to test whether there was an involvement of CB1 receptors, we investigated the effects of rimonabant in blocking the behavioural changes observed after genetic or pharmacological inhibition of FAAH.

2. Materials and methods

2.1. Subjects

The animals used in this study were male C57BL/6N mice, FAAH knock out ($FAAH^{-/-}$) mice and their WT littermates ($FAAH^{+/+}$), with a weight of 20–30 g and age of 3–4 months. The generation of $FAAH^{-/-}$ mice was described previously (Cravatt et al., 2001). Mutant mice were backcrossed 6 times into C57BL/6N background and heterozygous breedings were utilized. Tail biopsy and ear clip sampling were performed at an age of 6–10 weeks, and genotyping by PCR was performed as described (Cravatt et al., 2001). C57BL/6N mice were bred in the same animal facility as FAAH-deficient mice and were also tailed and ear marked. All animals were housed in a temperature- and humidity-controlled room where food and water were available ad libitum. Light phase was from 7:00 to 19:00. One week prior to behavioural analysis, mice were separated and single housed.

2.2. Drugs

Powdered diazepam (Sigma) and rimonabant (SR141716; NIMH Chemical Synthesis and Drug Supply Program) were suspended in 5% polyoxyethylene-sorbitan monooleate (Tween 80) followed by 0.9% NaCl solution (saline). URB597 (Cayman) was dissolved in a mixture of DMSO, ethanol and saline solution (1:1:18). All drugs were injected intraperitoneally (i.p.) in a volume of 10 ml/kg body weight.

2.3. Apparatus

All experiments were conducted between 9:00 and 13:00 h. The room was illuminated in a way that the intensity of light at the location of the apparatus was 200-lx (Luxmeter LX1108, Voltcraft, Germany). The experiments were recorded by a camera connected to a computer, where the program Smart (Panlab, Madrid) automatically evaluated the position of the animals and the distance moved.

The elevated plus maze (EPM) consisted of a cross-shaped plastic apparatus, elevated 100 cm from the floor, with 2 opposite open arms and 2 opposite enclosed arms. The floor of the arms was made of white plastic, 35 cm long and 6 cm wide and connected by a central platform of 6 × 6 cm. Walls in black plastic of 20 cm height surrounded the enclosed arms. The animals were placed into the centre of the apparatus, facing the enclosed arms, and were allowed to explore it during 5 min (Carobrez and Bertoglio, 2005). The percentage of time and entries in the open arms were calculated in relation to the total values for the open and enclosed arms through the following formula: % open arms = 100 × open/(open + enclosed).

The light dark test (LDT) was performed in a box (38 cm wide, with walls of 26 cm height) divided in a lit compartment (in white plastic, 26 cm in length; without a roof) and a dark compartment (in black plastic, 13 cm in length; with a roof). Lit and dark compartments were directly connected by a small entrance (5 × 5 cm). The animals were placed in the middle of the lit compartment and allowed to explore the apparatus during 5 min, starting with the first entry to the dark compartment (Bourin and Hascoet, 2003). The percentage of time spent in the lit was calculated as time (s) in this compartment divided by the total time, as follows: % time in the lit = 100 × time in the lit/300.

The open-field consisted of a squared transparent plastic apparatus (40 × 40 × 40 cm). The animals were placed in the centre and allowed to explore it during 5 min (Sousa et al., 2006).

2.4. Procedures

To avoid interferences in the baseline levels of anxiety or exploratory activity, the animals were not exposed twice to the tests – different batches of animals were used in each experiment. The behaviour of WT and $FAAH^{-/-}$ mice

was investigated in the open-field, in the EPM and in the LDT. First, experiments were conducted with these animals without any treatment. In another series of experiments, FAAH$^{-/-}$ and WT mice received either vehicle or rimonabant (3 mg/kg) injections 30 min before the tests. In the experiments with C57BL/6N mice, the animals were treated with vehicle or URB597 (1 mg/kg) 2 h before the tests (Naidu et al., 2007) with no further treatment or with rimonabant (3 mg/kg) or vehicle injection 30 min before the tests.

2.5. Statistics

The percentage of entries and time spent in the open arms as well as the number of entries in the enclosed arms of the EPM, the percentage of time spent in the lit compartment in the LDT and the total distance moved in the open field were analyzed by *t*-test or 2-way analysis of variance (ANOVA), as appropriate. Whenever an interaction between factors was found, post-hoc comparisons were performed with Bonferroni test. In addition, when a reduction in the number of entries in the enclosed arms of the EPM was detected, the percentage of entries in the open arms was re-analyzed by analysis of covariance (ANCOVA), with the number of entries in the enclosed arms as a co-variable. The data are presented as mean ± SEM, and the level of significance was set at a *p*-value of 0.05.

3. Results

3.1. Pharmacological validation of the animal models of anxiety

As positive controls for the EPM and the LDT, wild-type male C57BL/6N mice received an injection of the anxiolytic diazepam (2 mg/kg) or its vehicle 30 min prior to the experiments. The effect of diazepam on locomotion was evaluated in an open-field, where no significant change in the total distance moved was observed (Table 1). In the EPM, diazepam induced an increase in the percentage of entries (vehicle $14.76 \pm 6.68\%$ and diazepam $66.75 \pm 11.01\%$; $t_{14} = 4.03$, $p = 0.0012$; $n = 8$/group) and in the time spent (vehicle $10.90 \pm 5.69\%$ and diazepam $70.92 \pm 12.61\%$; $t_{14} = 4.33$, $p = 0.0007$) in the open arms. Its effect on the total number of entries in the enclosed arms is presented in Table 2. In the LDT, diazepam induced an increase in the percentage of time spent in the lit compartment (vehicle $15.84 \pm 2.57\%$ and diazepam $37.75 \pm 7.76\%$; $t_{15} = 2.75$, $p = 0.01$; $n = 8, 9$).

3.2. Anxiolytic-like effects after genetic inactivation of FAAH

In the first experiment, we aimed to analyze the phenotypes of WT and FAAH$^{-/-}$ in the EPM. Therefore, mice were exposed to the apparatus without receiving any injection. As depicted in Fig. 1, FAAH$^{-/-}$ mice entered more often ($t_{18} = 2.21$; $p = 0.04$; upper panel) and spent significantly more time ($t_{18} = 2.39$; $p = 0.02$; lower panel) in the open arms, consistent with a phenotype characterized by a reduced anxiety-like behaviour. WT and FAAH$^{-/-}$ did not differ regarding the distance moved in the open field (Table 1) or the number of entries in the enclosed arms of the EPM (Table 2). Next, as presented in Fig. 2, it was tested whether the difference between the genotypes is prevented by the CB1 receptor antagonist rimonabant (3 mg/kg). For the percentage of entries in the open arms, 2-way ANOVA did not reveal any interaction between genotype and drug factors ($F_{1,36} = 1.1$; NS). However, there was a significant effect of genotype ($F_{1,36} = 5.28$; $p = 0.02$) and drug ($F_{1,36} = 18$; $p = 0.0001$). Since there was no interaction between these factors, no post-hoc test was performed. However, for the percentage of time spent in the open arms, there was an borderline interaction between the genotype and drug factors ($F_{1,36} = 3.83$; $p = 0.05$). There was no genotype effect ($F_{1,36} = 2.15$; NS), but a significant drug effect ($F_{1,36} = 4.97$; $p = 0.03$). In this case, a post-hoc analysis was performed revealing that the vehicle-treated FAAH$^{-/-}$ mice spent significantly more time in the open arms as compared to all the other groups. As for the number of entries in the enclosed arms, there was no interaction between factors, neither a genotype effect (Table 2). However, rimonabant markedly reduced this parameter, regardless the genotype (Table 2). File (1992) proposed that, in these cases, the percentage of entries in the open arms should be re-analyzed by analysis of co-variance (ANCOVA), considering the number of entries in the enclosed arms as a co-variable. Accordingly, we performed a 2-way ANCOVA and the result of this analysis was similar to the former 2-way ANOVA. The interaction between factors did not change markedly, and there was still a tendency towards a significance ($F_{1,35} = 3.59$; $p = 0.06$). Again, no genotype effect was seen ($F_{1,35} = 2.41$; N.S.) and

Table 1
Total distance moved during 5 min assessed in the open field for the different mice and treatments employed in the present study ($n = 7–8$/group)

Experimental groups	Distance moved, Mean ± S.E.M.	Statistics (*t* test or 2-way ANOVA)
Vehicle (Veh)	1378 ± 107.7	$t(14) = 0.9032$; N.S.
Diazepam (2 mg/kg)	1196 ± 169.7	
WT	912.6 ± 163.3	$t(12) = 1.218$; N.S.
FAAH$^{-/-}$	1154 ± 112.9	
WT + Veh	616.5 ± 145.1	Interaction: F(1,28) = 0.92; N.S.
FAAH$^{-/-}$ + Veh	964.9 ± 93.6	Phenotype: F(1,28) = 2.63; N.S.
WT + Rimonabant (3 mg/kg)	566.5 ± 149.1	Drug: F(1,28) = 1.77; N.S.
FAAH$^{-/-}$ + Rimonabant	655.8 ± 143.7	
Veh + Veh	1683 ± 106.8	Interaction: F(1,28) = 2.03; N.S.
URB597 (1 mg/kg) + Veh	1570 ± 139.2	Veh × URB: F(1,28) = 0.43; N.S.
Veh + Rimonabant (3 mg/kg)	1587 ± 171.6	Veh × Rimonabant: F(1,28) = 0.59; N.S.
URB597 + Rimonabant	1890 ± 158.3	

Table 2
Total number of entries in the enclosed arms of the EPM ($n = 6–10$/group)

Experimental groups	Number of entries in the enclosed arms, Mean ± S.E.M.	Statistics (t test or 2-way ANOVA)
Vehicle (Veh)	13.25 ± 1.51	$t(14) = 1.91$; N.S.
Diazepam (2 mg/kg)	9.38 ± 1.36	
WT	12.90 ± 1.17	$t(18) = 1.31$; N.S.
FAAH$^{-/-}$	16.10 ± 16.15	
WT + Veh	10.4 ± 2.47	Interaction: F(1,36) = 0.73; N.S.
FAAH$^{-/-}$ + Veh	10.5 ± 2.11	Phenotype: F(1,36) = 0.70; N.S.
WT + Rimonabant (3 mg/kg)	2.9 ± 1.23	Drug: F(1,36) = 13.04; p = 0.0009
FAAH$^{-/-}$ + Rimonabant	4.3 ± 1.5	
Veh	11.33 ± 1.05	$t(10) = 0.90$; N.S.
URB597 (1 mg/kg)	10.01 ± 1.03	
Veh + Veh	13.50 ± 2.52	Interaction: F(1,28) = 1.11; N.S.
URB597 (1 mg/kg) + Veh	15.50 ± 0.87	Veh × URB: F(1,28) = 0.04; N.S.
Veh + Rimonabant (3 mg/kg)	17.38 ± 2.78	Veh × Rimonabant: F(1,28) = 0.38; N.S.
URB597 + Rimonabant	14.50 ± 2.58	

a drug effect was still present ($F_{1,35} = 6.45$; $p = 0.02$). Thus, although rimonabant significantly reduced entries into the enclosed arms, this effect may have a modest contribution to the changes in the percentage of time in the open arms. No changes in distance moved were found in the open field (Table 1).

To further explore the phenotype of the FAAH$^{-/-}$ mice, in a next series of experiments the WT and FAAH$^{-/-}$ mice were compared in another model of anxiety, the LDT. WT and FAAH$^{-/-}$ mice were exposed to the apparatus without receiving any injection. Supporting the results obtained in the EPM, a reduced anxiety-like behaviour was detected in FAAH$^{-/-}$ mice (Fig. 3). They spent significantly more time in the lit compartment as compared to the WT group ($t_{25} = 2.06$; $p = 0.04$). Finally, it was tested whether this difference is also prevented by rimonabant, as observed in the EPM. As depicted in Fig. 4, no interaction between the genotype and drug factors were found for the percentage of time spent in the lit compartment ($F_{1,34} = 2.73$; NS). However, there was a significant difference between the genotypes ($F_{1,34} = 6.31$; $p = 0.02$), further indicating a reduced anxiety-like behaviour in the FAAH$^{-/-}$ mice. There was also a significant drug effect ($F_{1,34} = 4.42$; $p = 0.04$), indicating that rimonabant was able to reduce the exploration of the lit compartment.

3.3. Anxiolytic-like effects after pharmacological inhibition of FAAH

In order to further strengthen the results obtained with FAAH$^{-/-}$ mice, we have also adopted a pharmacological approach to inhibit the FAAH enzyme. C57BL/6N mice were treated with vehicle or the FAAH inhibitor URB597 (1 mg/kg) and exposed to the EPM two hours later (Fig. 5). A significant increase both in the number of entries ($t_{10} = 2.88$; $p = 0.01$) and in the time spent ($t_{10} = 1.4$; $p = 0.0058$) in the open arms was detected. Next, to check for the involvement of CB1 receptors, the effect of rimonabant (3 mg/kg) was investigated (Fig. 6). A 2-way ANOVA was performed considering the following experimental factors: treatment with URB597 or its respective vehicle (URB factor) and treatment with rimonabant or its respective vehicle (rimonabant factor). For the percentage of entries in the open arms (Fig. 6, upper panel), a significant interaction between these factors was detected ($F_{1,28} = 5.37$; $p = 0.04$). There was no significant effect neither for the URB factor ($F_{1,28} = 0.21$; NS) nor for the rimonabant factor ($F_{1,28} = 0.03$; NS). Post-hoc analysis did not found any difference between particular groups. For the percentage of time spent in the open arms (Fig. 6, lower panel), a significant interaction between these factor was observed ($F_{1,28} = 4.42$; $p = 0.04$). Furthermore, there was an significant effect of URB ($F_{1,28} = 4.59$; $p = 0.04$), but not of the rimonabant factor ($F_{1,28} = 0.89$; NS). Post-hoc analysis revealed a significant increase in the time spent in the open arms only for the group treated with vehicle-URB597, as compared to the vehicle-vehicle group. Altogether, these pharmacological data mimic those observed with the FAAH$^{-/-}$ mice, as they indicate that FAAH inhibition induces an anxiolytic-like effect mediated by the CB1 receptor. Neither drug changed in locomotion in the open field (Table 1). In addition, as presented in Table 2, there were no changes in the total number of entries in the enclosed arms of the EPM.

Finally, we tested the effect of URB597 (1 mg/kg) in the LDT. C57BL/6N mice were treated with vehicle or URB597 (1 mg/kg) and exposed to the test 2 h later (Fig. 7). Contrary to the EPM, no significant effect was observed in this model of anxiety ($t_{20} = 1.4$; NS).

4. Discussion

The present study shows that FAAH$^{-/-}$ mice exhibit reduced anxiety-like behaviour in two experimental models. Since this phenotype was reversed after injection of the CB1 antagonist rimonabant, it is likely that elevated levels of eCBs acting via CB1 receptors are responsible for the

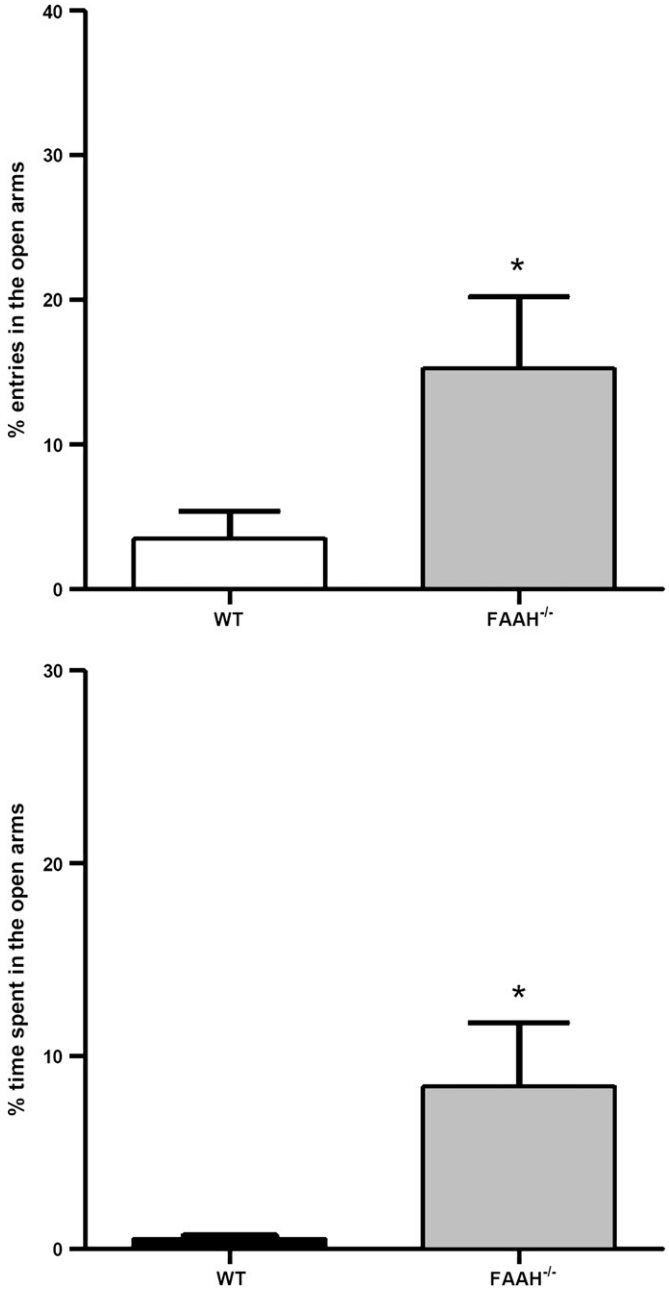

Fig. 1. Behaviour of FAAH$^{-/-}$ mice in the elevated plus maze. FAAH$^{-/-}$ mice entered more frequently (upper panel) and spent a longer time (lower panel) in the open arms as compared to their WT littermates, indicating a reduced anxiety-like behaviour (*$p < 0.05$; $n = 10$/group).

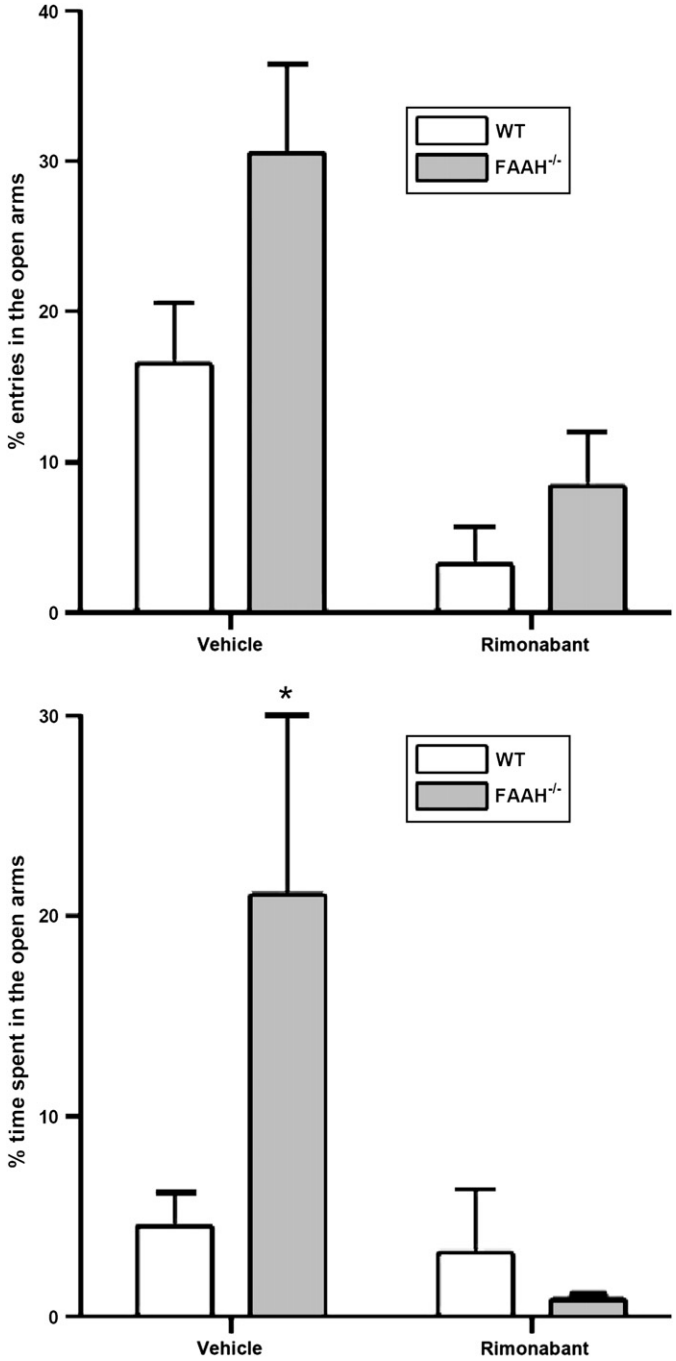

Fig. 2. Effects of the CB1 antagonist rimonabant on anxiety-like behaviour of WT and FAAH$^{-/-}$ mice in the elevated plus maze. Rimonabant (3 mg/kg) treatment in FAAH$^{-/-}$ mice was able to prevent the increase in the number of entries (upper panel) and in the time spent (lower panel) in the open arms, indicating increased signalling via CB1 receptors in FAAH$^{-/-}$ mice (*$p < 0.05$ compared to all the other groups; $n = 10$/group).

decreased anxiety in animals lacking FAAH. Furthermore, an anxiolytic-like effect was observed in C57BL/6N mice after injection of the FAAH inhibitor URB597, an effect blocked by rimonabant, and, thus, also mediated by CB1 receptors. No differences in spontaneous locomotion were observed in the open field, indicating that these results are not secondary to altered motor control in FAAH knock out or in drug-treated animals.

Significant differences between FAAH$^{-/-}$ mice and their WT littermates were observed in several experiments, supporting the consistency of this phenotype. These differences were observed both when the animals were exposed to the tests without any treatment and when they received vehicle injections. However, the baseline levels of anxiety were dissimilar between non-treated and vehicle-treated animals. One reason for this observation could be the stress caused by intraperitoneal injections. This procedure may have a major impact in the basal levels of anxiety, and even saline or ''sham'' injection may change baseline behaviour as compared to non-treated

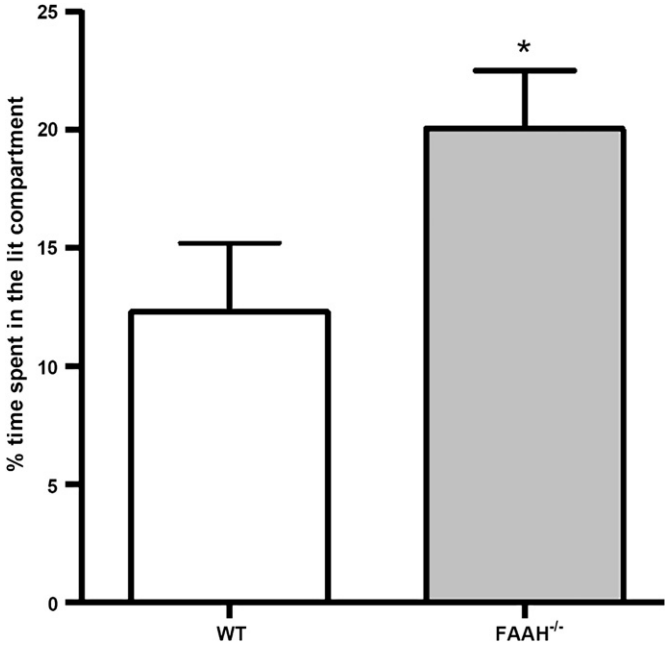

Fig. 3. Behaviour of FAAH$^{-/-}$ mice in the light-dark test. FAAH$^{-/-}$ mice spent a longer time in the lit compartment as compared to their WT littermates, indicating reduced anxiety-like behaviour (*$p < 0.05$; $n = 13$; 14).

animals (Carobrez and Bertoglio, 2005; Lapin, 1995). In addition, painful stimuli may increase the release of anandamide in brain regions that are also related to anxiety (Walker et al., 1999). This may also explain the differences observed in the present study.

The phenotype of FAAH$^{-/-}$ mice is congruent with previous pharmacological experiments demonstrating attenuation of anxiety without motor impairment by the FAAH-inhibitor URB597 in rats and mice (Kathuria et al., 2003; Patel and Hillard, 2006). Similar properties were reported in rats for the anandamide transporter inhibitor AM404 (Bortolato et al., 2006). Altogether, the available pharmacological and genetic evidence suggest that enhancing the activity of the endocannabinoid system would be a valuable strategy for the treatment of anxiety. Moreover, it supports the importance of this system in reducing the impact of aversive encounters of

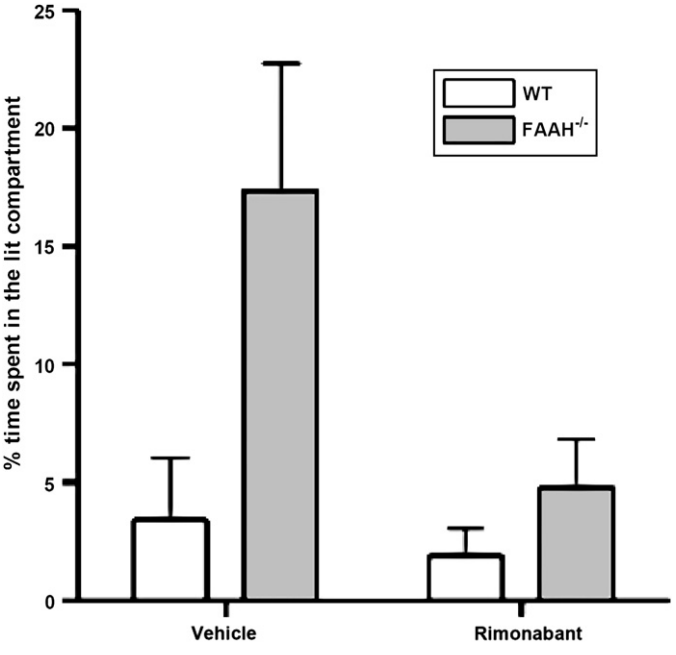

Fig. 4. Effects of the CB1 antagonist rimonabant on anxiety-like behaviour of WT and FAAH$^{-/-}$ mice in the light-dark test. Rimonabant (3 mg/kg) treatment reduced the time spent in the lit compartment in both groups of mice ($n = 10$; 9; 10; 9).

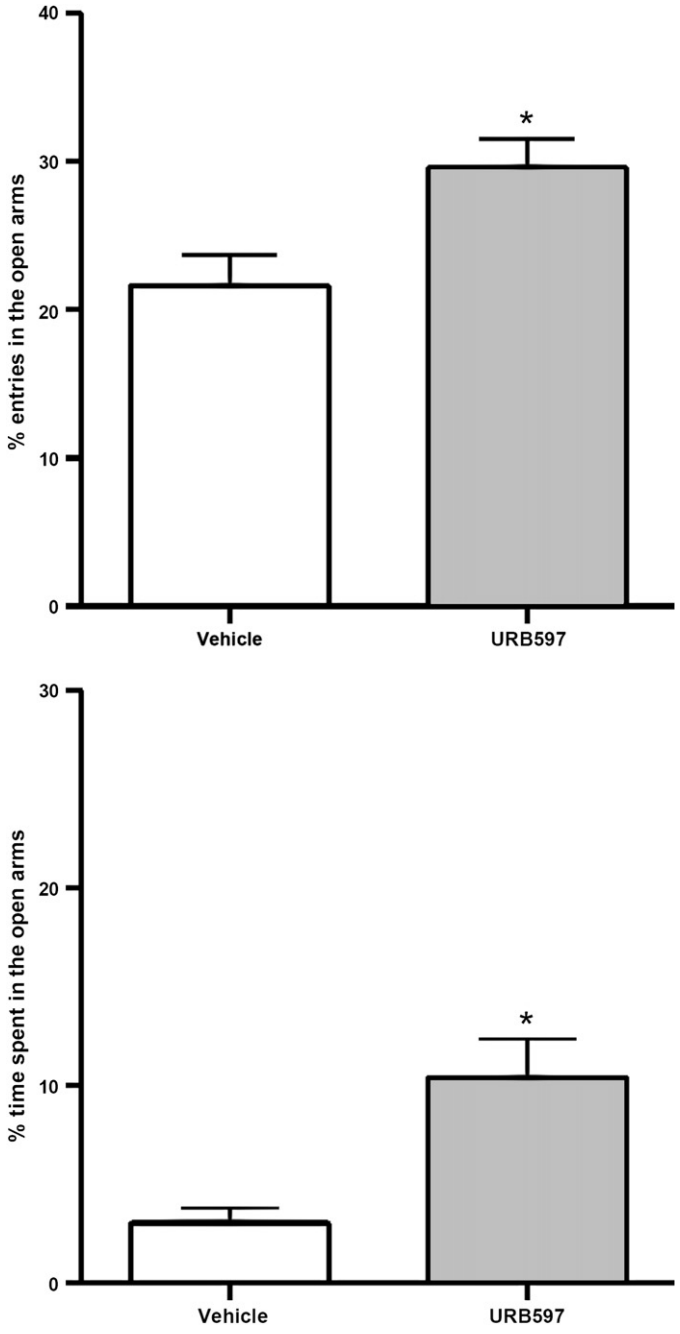

Fig. 5. Effect of pharmacological inhibition of FAAH on the behaviour of C57BL/6N mice in the elevated plus maze. Animals treated with URB597 (1 mg/kg) entered the open arms more frequently (upper panel) and spent a longer time (lower panel) in the open arms as compared to the vehicle-treated group, indicating an anxiolytic-like effect of URB597 (*$p < 0.05$; $n = 6$/group).

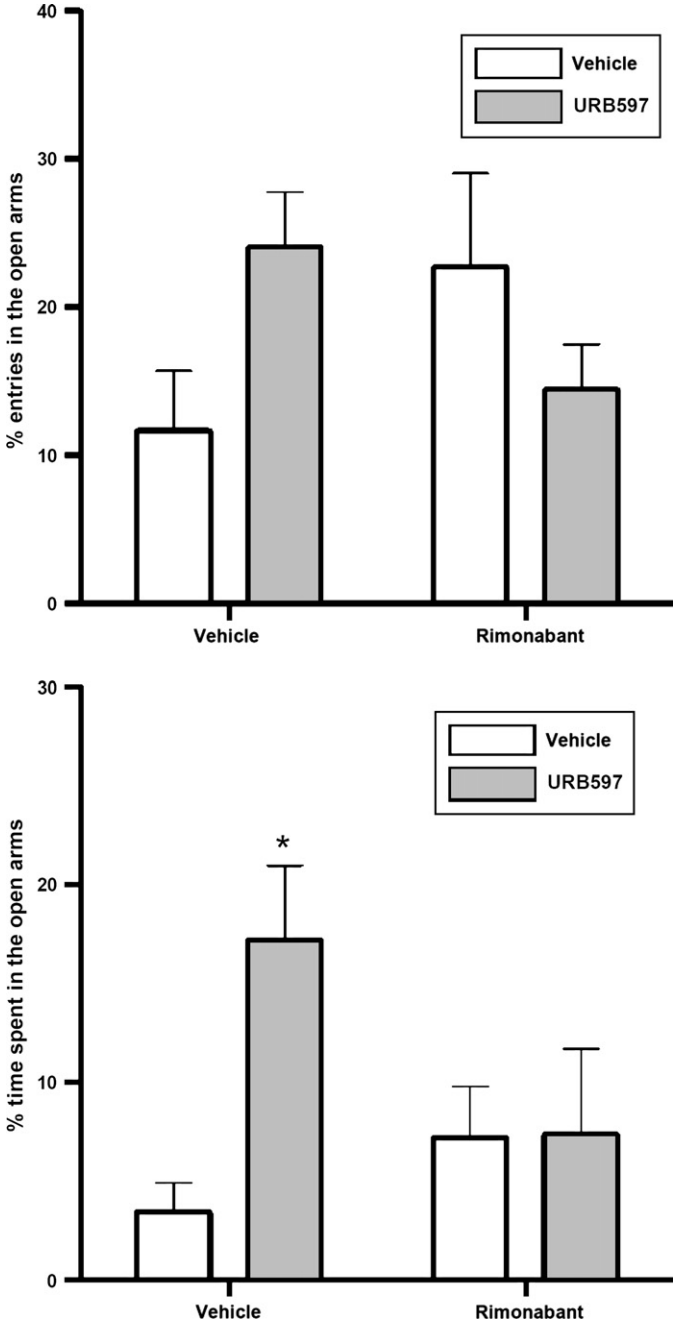

Fig. 6. Effect of the CB1 antagonist rimonabant on the anxiolytic-like activity of URB597 in C57BL/6N mice. Rimonabant (3 mg/kg) treatment was able to prevent the increase in the number of entries (upper panel) and in the time spent (lower panel) in the open arms induced by URB597 (1 mg/kg), indicating an involvement of CB1 receptors in the effect of URB597 ($*p < 0.05$ compared to all the other groups; $n = 8$/group).

either conditioned or unconditioned nature (Hill et al., 2006; Kamprath et al., 2006; Marsicano et al., 2002). Indeed, increased levels of eCBs were observed during aversive stimuli in key regions related to fear and anxiety responses, where this system may act on-demand to oppose the impact of stress (Marsicano et al., 2002). Based on this assumption, it is reasonable to suppose that an increased activation of CB1 receptors (due to higher levels of eCBs) can protect the FAAH$^{-/-}$ mice more efficiently from the aversive stimuli, as compared to wild-type mice.

The role of CB1 receptors in the modulation of anxiety is also supported by experiments with Δ^9-THC, which may induce anxiolytic effect in mice exposed to the LDT (Berrendero and Maldonado, 2002). However, anxiogenic effects have also been reported with this drug (Onaivi et al., 1990; Patel and Hillard, 2006). Generally, low doses of cannabinoids tend to be anxiolytic and higher doses tend to act on the opposite direction (Viveros et al., 2005). One possible explanation for this phenomenon is the presence of CB1 receptor in pre-synaptic terminals in several brain regions, modulating both excitatory (glutamate) and inhibitory (γ-aminobutyric acid, GABA) neurotransmissions (see Monory et al., 2006; and references therein). Therefore, cannabinoids interfere with systems that exert opposing activities on anxiety responses (Millan, 2003). Importantly, GABAergic terminals contain high levels of CB1 receptors, while glutamatergic terminals contain low levels (Marsicano and Lutz, 1999; Monory et al., 2006). Hence, low concentrations of cannabinoid drugs might be able to effectively activate a significant portion of CB1 receptors on glutamatergic terminals, while a much higher concentration would be needed to activate the same fraction of CB1 receptors on GABAergic terminals. For a better understanding of this subject, the molecular mechanisms and the brain regions underlying the behavioural effects of cannabinoids should be further investigated. One possible site for their anxiolytic activity could be the hippocampus, since this action is prevented by blockade of neurogenesis in this structure (Jiang et al., 2005). Another candidate is the dorsolateral periaqueductal gray, since injections of CB1 receptor agonists into this structure induce anxiolytic-like effects (Moreira et al., 2007). Understanding the mechanism by which the eCBs modulate these systems may help to clarify their role on anxiety-related behaviours.

The phenotype in FAAH$^{-/-}$ mice was reversed by rimonabant both in the EPM and in the LDT. This drug also blocked the effect of URB597 in C57BL/6N mice exposed to the EPM. Thus, an enhancement of CB1 receptor-mediated signalling, presumably due to higher levels of anandamide, seems to be responsible for the reduction in anxiety-related behaviours. However, it should be noted that in FAAH$^{-/-}$ and WT mice rimonabant reduced the number of entries in the enclosed arms of the EPM. To check whether this effect would be interfering with the percentage of time spent in the open arms, we re-analyzed this data by 2-way ANCOVA with the number of entries in the enclosed arms a co-variable. The result of this analysis was similar to the 2-way ANOVA. Thus, it may be concluded that although rimonabant markedly reduced the entries in the enclosed arms, this may have a minor contribution to the effects on the percentage of time spent in the open arms (File, 1992). In C57BL/6N mice, this drug did not change enclosed arms entries. Also, it failed to interfere with total distances moved by FAAH$^{-/-}$, WT or C57BL/6N mice in the open field. Consistent with our study, Kathuria et al. (2003) also showed that rimonabant blocked the anxiolytic effect induced by pharmacological inhibition of FAAH in rats, also

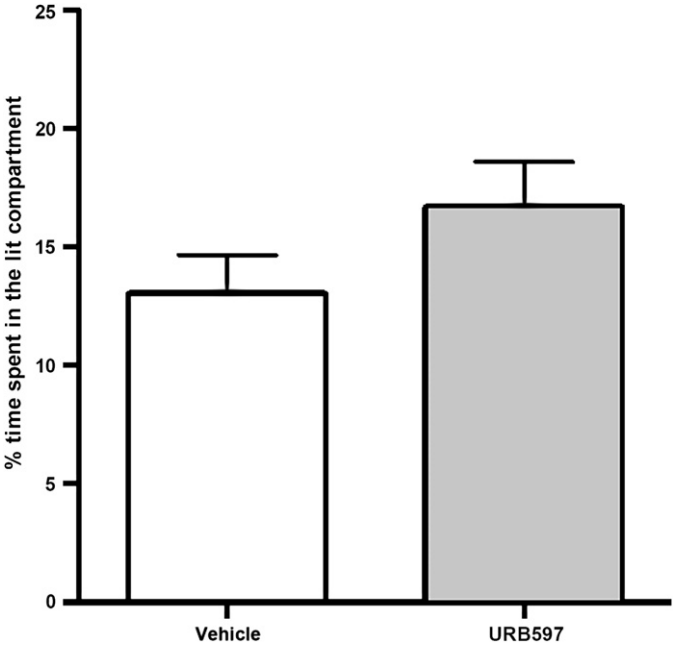

Fig. 7. Effect of the pharmacological blockade of FAAH on the behaviour of C57BL/6N mice in the light-dark test The animals treated with URB597 (1 mg/kg) did not spent a longer time in the lit compartment as compared to the vehicle treated group ($n = 10$; 12).

pointing to an involvement of CB1 receptors in the anxiolytic. These results are relevant because anandamide is a promiscuous neurotransmitter that may bind to other sites in the brain (Ross, 2003; Wise et al., 2007). In our study, apart from blocking the phenotype of FAAH$^{-/-}$ mice, rimonabant also inhibited the exploration of aversive environments in WT mice. This means that this CB1 antagonist induced an anxiogenic-like effect, in accordance with the possible existence of a modulatory tonus by endocannabinoids on anxiety. However, the same was not observed in C57BL/6N mice, in which rimonabant selectively prevented the anxiolytic effect of URB597, without any reduction in basal levels in vehicle-treated mice. These diverse responses have been observed after blockade of the CB1 receptors with either rimonabant or AM251. While in some cases, these drugs may induce no modification on measures of anxiety (Kathuria et al., 2003), others studies show anxiogenic effects (Navarro et al., 1997; Patel and Hillard, 2006; Rodgers et al., 2005). Studies with CB1-deficient mice also showed a complex behaviour. These mutants may have either increased levels of anxiety or no phenotype, depending on the environmental stimuli (Haller et al., 2004a; Martin et al., 2001).

Apart from CB1 receptors, an alternative site mediating some effects of anandamide is the TRPV1 receptor (Ross, 2003), which is also located in brain regions related to anxiety (Cristino et al., 2006). However, it is unlikely that an increased activation of this receptor would contribute to the anxiolytic-like phenotype of FAAH$^{-/-}$ mice. One reason is that the phenotype was blocked by rimonabant, as discussed above. Another reason is that, contrary to CB1 receptors, the pharmacological blockade of TRPV1 receptors induces anxiolytic, rather than anxiogenic responses (Kasckow et al., 2004). Also, TRPV1-deficient mice exhibit a phenotype characterized by reduced, rather than increased, levels of anxiety (Marsch et al., 2007). Altogether, these data support the notion that anandamide might balance anxiety by exerting actions in opposite directions via the activation of CB1 and TRPV1 receptors (Marsch et al., 2007).

At the time when the present experiments were being conducted, another study showed no phenotype of the FAAH$^{-/-}$ mice in the EPM (Naidu et al., 2007). Although the present results are apparently discrepant with the cited investigation, differences in genetic background (C57BL/6N versus C57BL/6J), animal housing as well in experimental context might explain these discrepancies. In fact, in the study by Naidu et al. (2007), the FAAH inhibitor URB597 was ineffective when the experiments were conducted under low light environment, although an anxiolytic-like effect was detected when illumination over the open arms of the EPM was increased. These differences may arise because the stress levels of the subjects and the experimental context are critical factors for detecting anxiolytic responses in the EPM (Mechiel Korte and De Boer, 2003). Such parameters might be particularly relevant in manipulations involving eCBs, which are supposed to act on-demand to counteract aversive responses (Marsicano et al., 2002; Kamprath et al., 2006). In line with this view, CB1 knock-out mice have a phenotype characterized by an increased anxiety-like behaviour, which is observed only when the experiments are conducted under high light intensity (Haller et al., 2004a). Therefore, different experimental environments may explain some apparent discrepancies in studies with the endocannabinoid system.

Finally, we adopted a pharmacological approach to further strengthen the results obtained with FAAH$^{-/-}$ mice. In accordance with previous data (Kathuria et al., 2003; Naidu et al., 2007; Patel and Hillard, 2006), the FAAH inhibitor induced an anxiolytic-like effect in rodents exposed to the EPM, which was prevented by rimonabant. However, while Rutkowska et al. (2006) detected an anxiolytic effect in mice exposed to the LDT after treatment with the FAAH inhibitor AACOCF3, no significant effect of URB597 was observed in this model in the present study, even though diazepam was effective as a positive control. A possible explanation for the discrepancy between the EPM and the LDT is that these models may generate different levels of aversion. While the LDT is based on the aversion for a lit environment, the EPM is based on the aversion for an open environment (Bourin and Hascoet, 2003; Carobrez and Bertoglio, 2005). It is possible that these models have different sensitivities to detect interventions in the endocannabinoid system. In addition to this lack of effect in the LDT, the effect of URB597 in the EPM was not as efficacious as that observed with the reference compound diazepam. One possible advantage of URB597 over diazepam could be its low potential to induce sedation and memory impairment. Also, the observation that it does not induce conditioned place-preference (Gobbi et al., 2005) suggests its low potential to be addictive. However, to date, it is not fully clear which side effects a long-term deficiency of FAAH activity

may cause because of the accumulation of other classes of lipids, such as N-acyl taurines (Saghatelian et al., 2006). Furthermore, a potential problem in humans is the recent finding of a second FAAH gene (FAAH-2), which is not present in rodents. FAAH-2 is, however, also inhibited by URB597 (Wei et al., 2006).

In conclusion, the present study shows that FAAH$^{-/-}$ mice have a phenotype characteristic of a reduced anxiety-like behaviour, as revealed by two different behavioural models. Furthermore, it suggests that this phenotype is due to an increased activation of CB1 receptors, possibly reflecting the increased levels of anandamide after FAAH inhibition. Finally, it further supports the anxiolytic-like activity of the FAAH inhibitor URB597. These results point to the enhancement of eCBs activity as a promising strategy for the alleviation of anxiety-related disorders.

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