Anandamide, the naturally occurring amide of arachidonic acid with ethanolamine, meets all key criteria of an endogenous cannabinoid substance: it is released on demand by stimulated neurons; it activates cannabinoid receptors with high affinity; and it is rapidly eliminated through a two-step process consisting of carrier-mediated transport followed by intracellular hydrolysis. Anandamide hydrolysis is catalyzed by the enzyme fatty acid amide hydrolase (FAAH), a membrane-bound serine hydrolase that also cleaves other bioactive fatty acid ethanolamides such as oleoylethanolamide and palmitylethanolamide. Mutant mice lacking the gene encoding FAAH cannot metabolize anandamide and, although fertile and generally normal, show signs of enhanced anandamide activity at cannabinoid receptors such as reduced pain sensation. This is suggestive that drugs targeting FAAH may heighten the tonic actions of anandamide, while possibly avoiding the multiple and often unwanted effects produced by Δ9-tetrahydrocannabinol (Δ9-THC) and other direct-acting cannabinoid agonists. An alternative approach may be to develop drugs that amplify the effects of endogenous cannabinoids by preventing their inactivation. Here we describe a class of potent, selective and systemically active inhibitors of fatty acid amide hydrolase, the enzyme responsible for the degradation of the endogenous cannabinoid anandamide. Like clinically used anti-anxiety drugs, in rats the inhibitors exhibit benzodiazepine-like properties in the elevated zero-maze test and suppress isolation-induced vocalizations. These effects are accompanied by augmented brain levels of anandamide and are prevented by CB1 receptor blockade. Our results indicate that anandamide participates in the modulation of emotional states and point to fatty acid amide hydrolase inhibition as an innovative approach to anti-anxiety therapy.

Anandamide, the naturally occurring amide of arachidonic acid with ethanolamine, meets all key criteria of an endogenous cannabinoid substance: it is released on demand by stimulated neurons; it activates cannabinoid receptors with high affinity; and it is rapidly eliminated through a two-step process consisting of carrier-mediated transport followed by intracellular hydrolysis. Anandamide hydrolysis is catalyzed by the enzyme fatty acid amide hydrolase (FAAH), a membrane-bound serine hydrolase that also cleaves other bioactive fatty acid ethanolamides such as oleoylethanolamide and palmitylethanolamide. Mutant mice lacking the gene encoding FAAH cannot metabolize anandamide and, although fertile and generally normal, show signs of enhanced anandamide activity at cannabinoid receptors such as reduced pain sensation. This is suggestive that drugs targeting FAAH may heighten the tonic actions of anandamide, while possibly avoiding the multiple and often unwanted effects produced by Δ9-tetrahydrocannabinol (Δ9-THC) and other direct-acting cannabinoid agonists. To test this hypothesis, potent, selective and systemically active inhibitors of intracellular FAAH activity are needed. However, most current inhibitors of this enzyme lack the target selectivity and biological availability required for in vivo studies, whereas newer compounds, though promising, have not yet been characterized. Thus, the therapeutic potential of FAAH inhibition remains essentially unexplored.

Lead identification and optimization

Despite its unusual catalytic mechanism, FAAH is blocked by a variety of serine hydrolase inhibitors, including compounds with activated carbonyls. Therefore we examined whether esters of carboxylic acid such as the anti-cholinesterase agent carbaryl (compound 1; Table 1) might inhibit FAAH activity in rat brain membranes. Although compound 1 was ineffective, its positional isomer (compound 2) produced a weak inhibition of FAAH (half-maximal inhibitory concentration (IC50) = 18.6 ± 0.7 µM; mean ± s.e.m., n = 3), which was enhanced by replacing the N-methyl substituent with a cyclohexyl group (compound 3; IC50 = 324 ± 31 nM). The aryl ester (compound 4), the benzyloxyphenyl group of which can be regarded as an elongated bioisosteric variant of the naphthyl moiety of compound 2, inhibited the activity of FAAH with a potency (IC50) = 396 ± 63 nM). The aryl ester 4, the benzyloxyphenyl group of which can be regarded as an elongated bioisosteric variant of the naphthyl moiety of compound 2, inhibited the activity of FAAH with a potency (IC50) = 396 ± 63 nM) equivalent to that of compound 3. A conformational analysis of compound 4 revealed families of accessible conformers differing mainly in the torsion angle around the O–CH2 bond, with substituents in anti or gauche conformations (data not shown). As the latter conformations more closely resembled the shape of the naphthyl derivative 3, we hypothesized that they might be responsible for the interac-
and URB597 (10 nM) promote accumulation of non-metabolized [3H]anandamide in neurons, whereas the anandamide transport inhibitor AM404 (AM, 10 µM) reduces it. V, vehicle.

d Release of non-metabolized [3H]anandamide from neurons treated with URB597 (10 nM) during a 15-min incubation in the absence or presence of AM404 (AM, 10 µM). e, Time course of [3H]anandamide release from neurons treated with URB597 (10 nM). *P < 0.05; **P < 0.01 versus vehicle-treated neurons; ANOVA with Tukey’s post-hoc test (n = 4–8). V, vehicle; ■, treatments. Error bars, s.e.m.

**Table 1 Structures of selected carbamate inhibitors of FAAH activity**

| R | R₁ | IC₅₀ (nM) |
|---|---|---------|
| 1 | | >100,000 |
| 2 | CH₃ | 18,600 ± 708 |
| 3 | c-C₆H₄ | 324 ± 31 |
| 4 | n-C₆H₄ | 396 ± 63 |
| 5 | c-C₆H₄ | 63 ± 9 |
| 6 | c-C₆H₄ | 4.6 ± 1.6 |
| 7 | URB597 | >100,000 |
| 8 | p-FC₆H₄ | >100,000 |

Values reported are the concentrations required to inhibit FAAH activity by 50% (IC₅₀, nM), and are expressed as the mean ± s.e.m. of at least three independent experiments. They were calculated from concentration-response curves, by using non-linear regression analysis as implemented in the Prism 2.0 software package.
cholinesterase, horse plasma butyryl cholinesterase and rat brain monoglyceride lipase (MGL; Table 2). The lack of MGL inhibition is particularly noteworthy in light of the proposed role of this enzyme in the biological inactivation of 2-arachidonoylglycerol (2-AG), another endogenous cannabinoid present in brain.18–20 Furthermore, URB532 and URB597 had no effect on anandamide transport in human astrocytoma cells or on the binding of a high-affinity ligand to CB1 and CB2 receptors (Table 2). In addition, URB532 (10 μM) did not significantly interact with a panel of 21 receptors, ion channels and neurotransmitter transporters, which included adenosine A1, A2a, A2b and A3; adrenergic α1, α2, β1 and β2; dopamine D1 and D2; glutamate N-methyl-D-aspartate; γ-aminobutyric acid (GABA), agonist site; histamine H1; opiate μ, muscarinic M2; and brain nicotinic receptors (data not shown). This high selectivity for FAAH encouraged us to examine the effects of URB532 and URB597 in live animals.

FAAH inhibition in vivo

Intraperitoneal (i.p.) injection of either URB532 or URB597, but not the inactive analog 8, produced a profound, dose-dependent inhibition of brain FAAH activity (Fig. 2a). In six experiments, half-maximal inhibition was reached at 0.60 ± 0.09 mg kg⁻¹ of URB532, and 0.150 ± 0.007 mg kg⁻¹ of URB597. The discrepancy between potency ratios of URB532 and URB597 in vitro (100-fold) and in vivo (4-fold) presumably reflects differences in bioavailability or brain penetration of the two compounds. After injection of a maximal dose of URB597 (0.3 mg kg⁻¹, i.p.), FAAH inhibition was rapid in onset (<15 minutes), persistent (>6 hours; Fig. 2b) and accompanied by significant elevations in the brain levels of anandamide (Fig. 2c) and other fatty acid ethanolamides that are substrates for FAAH (in pmol g⁻¹ of tissue at 2 hours after injection: oleoylethanolamide, vehicle, 137.0 ± 14.3, URB597 (0.3 mg kg⁻¹), 725.3 ± 28.6; palmitoylethanolamide, vehicle, 259.1 ± 15.0, URB597, 1,324 ± 395; n = 8–15). Parallel changes in FAAH activity and fatty acid ethanolamide levels were also measured in various peripheral tissues (data not shown). In agreement with the lack of MGL inhibition noted in our in vitro experiments (Table 2), URB597 did not change the brain content of 2-AG (Fig. 2d).

As previously observed in Faah⁻/⁻ mice, FAAH inhibition was associated with increased sensitivity to the administration of exogenous anandamide. Accordingly, URB597 (0.3 mg kg⁻¹, i.p.) intensified and prolonged the decrease in body temperature elicited by a subthreshold dose of anandamide (5 mg kg⁻¹, i.p.), whereas it had no effect when injected alone (Figs. 2e and f). Underscoring the role of CB1 receptors in this response, the effect of anandamide plus URB597 was prevented by the CB1 antagonist SR141716A (rimonabant; Figs. 2e and f).

Pharmacological properties of FAAH inhibitors in vivo

Although URB532 and URB597 increased brain anandamide lev-

---

**Table 2 Analysis of selected FAAH inhibitors in vitro**

| Compound | AChE | BCh | MGL | AT | CB1 | CB2 |
|----------|------|-----|-----|----|-----|-----|
| URB532  | >100, SI ≥ 333 | >100, SI ≥ 333 | >30, SI ≥ 100 | >300, SI ≥ 1,000 | >300, SI ≥ 1,000 | >300, SI ≥ 1,000, SI ≥ 2,5000 |
| URB597  | >100, SI ≥ 2,5000 | >100, SI ≥ 25,000 | >30, SI ≥ 7,500 | >30, SI ≥ 7,500 | >100, SI ≥ 2,5000 | >100, SI ≥ 2,5000 |

Values indicate the maximal concentrations of FAAH inhibitor tested on each target (in μM) and their corresponding selectivity index (SI). The SI is the ratio of maximal inhibitor concentration tested/IC₅₀ for FAAH (from Table 1).
els, they did not mimic the spectrum of pharmacological responses produced by exogenous anandamide. Systemic doses of URB532 (10 mg kg\(^{-1}\), i.p.) or URB597 (0.3 mg kg\(^{-1}\), i.p.) that maximally blocked FAAH activity produced no catalepsy (rigid immobility), hypothermia or hyperphagia (increased food intake), three typical signs of CB1 receptor activation\(^1\) (data not shown). However, the compounds exerted moderate anti-nociceptive actions in the mouse hot-plate test, which measures response to noxious thermal stimuli. In this model, URB532 and URB597 significantly lengthened response latencies at a dose of 0.5 mg kg\(^{-1}\) (Figs. 3a and b), but not 0.1 mg kg\(^{-1}\) (data not shown). These effects were prevented by a dose of the CB1 antagonist rimonabant (0.2 mg kg\(^{-1}\), intravenous (i.v.); Figs. 3a and b), which caused no hyperalgesia when administered alone (data not shown). Our results corroborate those obtained in Faah\(^{-/-}\) mice\(^2\), indicating that acute disruption of FAAH activity results in a mild CB1-mediated anti-nociception, but no hypothermia or catalepsy.

**Anxiolytic effects of FAAH inhibitors**

To identify intrinsic actions of anandamide that might be significantly magnified by FAAH inhibition, we turned, for three reasons, to the regulation of emotional reactivity. First, CB1 receptors are expressed at high levels in brain regions such as the amygdala which are implicated in the control of anxiety and fear\(^{21-23}\). Second, acute administration of cannabinoid drugs produces emotional responses in rodents\(^1\) and humans\(^{10,24}\). Third, pharmacological\(^{25,26}\) or genetic\(^{27-29}\) disruption of CB1 receptor activity elicits anxiety-like behaviors in rodents, suggestive of the existence of an intrinsic anxiolytic tone mediated by endogenous cannabinoids.

We used two pharmacologically validated animal models of anxiety: the elevated zero-maze test and the isolation-induced ultrasonic emission test. The zero maze consists of an elevated annular platform with two open and two closed quadrants and the test is based on the conflict between an animal's instinct to explore its environment and its fear of open spaces, where it may be attacked by predators\(^{28,29}\). Clinically used anxiolytic drugs such as the benzodiazepines increase the proportion of time spent in, and the number of entries made into, the open compartments. Similarly, URB532 (0.1–10 mg kg\(^{-1}\), i.p.) and URB597 (0.05–0.1 mg kg\(^{-1}\), i.p.) evoked anxiolytic-like responses at doses that corresponded to those required to inhibit FAAH activity in vivo (F = 24.7, df = 4/41, P < 0.001; F = 7.7, df = 2/27, P < 0.01; Figs. 4a and b). In keeping with an involvement of endogenous anandamide, the anxiolytic-like effects of both compounds were attenuated by a non-anxiogenic dose of rimonabant (2 mg kg\(^{-1}\), i.p.; F = 14.87, df = 3/28, P < 0.001; F = 15.2, df = 3/28, P < 0.001; Figs. 4c and d). Moreover, the effects were apparently dissociated from overall changes in motor behavior. Indeed, although URB532 elicited in adult rats a modest decrease in ambulation (which was also antagonized by rimonabant; data not shown), it did so at doses that were higher than those needed to cause anxiety (≥10 mg kg\(^{-1}\); F = 3.57, df = 2/22, P < 0.05; Fig. 4e). We confirmed this dissociation by testing URB532 and URB597 in the ultrasonic vocalization emission model, which measures the
number of stress-induced vocalizations emitted by rat pups removed from their nest. As seen with anxiolytic drugs, the FAAH inhibitors reduced ultrasonic calls (F = 10.8, df = 5/33, P < 0.001; F = 19.3; df = 4/25, P < 0.001; Figs. 4f and g) at doses (URB532, 1 and 5 mg kg⁻¹; URB597, 0.1 mg kg⁻¹) that had no effect on pup movement (data not shown). These anxiolytic-like responses were blocked by rimonabant (2 mg kg⁻¹; Figs. 4f and g).

Discussion
We have developed a new class of agents that prevents anandamide inactivation by targeting the intracellular enzymatic activity of FAAH. URB597, the most potent member of this class, inhibited FAAH activity with an IC₅₀ value of 4 nM in brain membranes and 0.5 nM in intact neurons, and an ID₅₀ value of 0.15 mg kg⁻¹ after systemic administration in the rat. This compound had much greater selectivity for FAAH than other cannabinoids-related targets, including cannabinoid receptors (selectivity index, >25,000) and MGL, an enzyme involved in the deactivation of the endogenous cannabinoid ester 2-AG (selectivity index, >7,500). Such target discrimination was matched by a lack of overt cannabimimetic effects in vivo. Thus, at doses that almost abolished FAAH activity and substantially raised brain anandamide levels, URB597 and its analog URB532 did not evoke catalepsy, reduce body temperature or stimulate feeding, three key symptoms of cannabinoid intoxication in the rodent. Nevertheless, the compounds did elicit marked anxiolytic-like responses, which paralleled their ability to inactivate FAAH and were prevented by the CB1 receptor antagonist rimonabant. We interpret these findings to indicate that URB597 and URB532 may selectively modulate anxiety-like behaviors by enhancing the tonic actions of anandamide on a subset of CB1 receptors which normally might be engaged in controlling emotions. Forebrain sites that might be implicated in such actions include the basolateral amygdala, the anterior cingulate cortex and the prefrontal cortex, all key elements of an “emotion circuit” that contains high densities of CB1 receptors. CB1 receptors in these structures are located to the axon terminals of a subpopulation of GABAergic interneurons, which also express the peptide cholecystokinin (CCK). The anxiogenic properties of CCK and the ability of CB1 agonists to inhibit K⁺-evoked CCK release from hippocampal slices indicate that interactions between this peptide and anandamide may participate in the control of anxiety.

In addition to their anxiolytic-like actions, URB597 and URB532 exerted modest anti-nociception in a model of acute pain, which also was sensitive to CB1 receptor blockade. These findings are similar to those reported for Faah⁻/⁻ mice and support the proposed roles of anandamide in the intrinsic modulation of pain. However, as emotional states may strongly influence pain sensation, it is possible that anxiolysis might have contributed to the mild anti-nociceptive effects of the FAAH inhibitors.

URB597 and URB532 increased brain anandamide levels without modifying those of the second endogenous cannabinoid, 2-AG. It is therefore likely that the pharmacological actions of these compounds, which are sensitive to the CB1 antagonist rimonabant, are primarily due to anandamide accumulation. But the FAAH inhibitors also produced large elevations in the levels of two anandamide analogs, palmitoylethanolamide and oleoylthanolamide, whose biological effects are independent of CB1 receptors. Thus, we cannot exclude the possibility that additional properties of URB597 and URB532, mediated by these fatty ethanolamides, remain to be discovered.

Our results define a novel class of inhibitors of FAAH activity, which enhance endogenous anandamide signaling without directly interacting with cannabinoid receptors. The behavioral profile of these agents—characterized by anxiolysis and mild analgesia—reveals a key role for anandamide in the regulation of emotional states and indicates a new mechanistic approach to anti-anxiety therapy.

Methods
Animals and cells. We used Wistar rats (200–350 g) and Swiss mice (20 g). All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and those of the Italian Ministry of Health (D.L. 116/92). We prepared cultures of cortical neurons from 18-day-old Wistar rat embryos and maintained them as described. We purchased astrocytoma cells from American Type Culture Collection (Manassas, Virginia).

Chemicals. Anandamide and related lipids were synthesized in the laboratory. SR141716A (rimonabant) was provided by RBI (Natick, Massachusetts) as part of the Chemical Synthesis Program of the National Institutes of Health. AM404 was from Tocris (Avonmouth, UK) and other drugs from Sigma.

Synthesis of inhibitors. N-Butylcarbamic acid 4-benzyloxyphenyl ester (URB532, compound 4) and 4-fluorophenylcarbamic acid 4-benzyloxyphenyl ester (compound 8) were obtained by treatment of 4-benzyloxyphenol with n-butylisocyanate and 4-fluorophenylisocyanate, respectively; with a catalytic amount of triethylamine in refluxing toluene. Similarly, cyclohexylcarbamic acid biphenyl-3-yl ester (compound 5), cyclohexylcarbamic acid 5-phenylpentyl ester (compound 7) and cyclohexylcarbamic acid 3′-carbamoyl-biphenyl-3-yl ester (URB597; compound 6) were synthesized by reacting cyclohexylisocyanate with 3-phenyl/phenol, 5-phenylpentan-1-ol and 3’-hydroxybiphenyl-3-carboxylic acid amide, respectively. The latter reactant was prepared as follows: 3-bromobenzoic acid amide, obtained by reaction of 3-bromobenzonitrile and sodium perborate, was coupled with methoxycarbonyl-boronic acid to give 3’-methoxybiphenyl-3-carboxylic acid amide, which was hydrolyzed with BB₃, to the desired 3’-hydroxybiphenyl-3-carboxylic acid amide. Detailed synthetic procedures and physicochemical data will be reported elsewhere.

Biochemical assays. We prepared cell fractions from brain homogenates and assayed membrane FAAH activity and cytosol MGL activity using anandamide[ethanolamine-1H] (60 Ci/mmol; American Radiolabeled Chemicals (ARC), St. Louis, Missouri) and 2-mono-oleoyl-glycerol-[glycerol-1,2,3-3H] (20 Ci/mmol; ARC, St. Louis, Missouri), respectively, as substrates. We conducted [3H]anandamide transport assays in human astrocytoma cells; CB1 and CB2 binding assays in rat cerebellar membranes and CB2-overexpressing CHO cells (Receptor Biology-Perkin Elmer, Wellesley, Massachusetts), respectively, using [3H]WIN-55212-2 (20 Ci/mmol) as a ligand; and cholinesterase assays with a commercial kit (Sigma) using purified enzymes (electric eel acetylcholinesterase type V-S and horse serum cholinesterase; both from Sigma). To measure anandamide transport and hydrolysis in cortical neurons, we preincubated cells with FAAH inhibitors for 10 min at 37 °C, before exposure to [3H]anandamide for 4 min. In some experiments, we stopped the reactions with cold Tris-Krebs buffer containing 0.1% bovine serum albumin (Type V, fatty acid free, Sigma), removed the cells by trypsin-EDTA treatment and extracted lipids with chloroform/methanol (1/1, vol/vol). We measured non-metabolized [3H]anandamide in the organic phase and metabolized [3H]anandamide (as [3H]ethanolamine) in the aqueous phase. In other experiments, after having exposed the neurons to [3H]anandamide for 4 min, we rinsed the cells and measured [3H]anandamide release as described above.

High-performance liquid chromatography/mass spectrometry. We extracted lipids with methanol-chloroform and fractionated them by column chromatography. Anandamide and other fatty acid derivatives were quantified by high-performance liquid chromatography/mass spectrometry.

Body temperature and catalepsy. We administered compounds (in saline/Tween/80/polyethylene glycol, 90/5/5, i.p.) immediately before tests. We measured body temperature with a rectal probe connected to a digital
thermometer (Physitemp Instruments, Clifton, New Jersey), and catalepsy as described.

Food intake. We administered URB597 (in DMSO/saline, 7:3, i.p.) 45 min before tests. We recorded food intake in free-feeding rats by using an automated system (Sciprio Inc., New York, New York). After 3-d acclimation, tests began at the onset of the dark phase and lasted for 24 h.

Anti-nociception. FAAH inhibitors (in polyethylene glycol/water, 1:1) and rimonabant (in saline) were tested in the mouse hot-plate assay, as described.

Anxiety and motor activity. We dissolved FAAH inhibitors and rimonabant in dimethylsulfoxide (DMSO)/saline (7:3 and 9:1, respectively). We administered FAAH inhibitors by i.p. injection 30 min before tests and rimonabant 30 min before FAAH inhibitors. The elevated zero-maze apparatus is described elsewhere. We placed the rats in a closed quadrant and video-recorded them for 5-min periods. Results are expressed as percent time in open quadrant/total time (percent time open). Results were analyzed by one-way ANOVA followed by Tukey’s test for individual between-group comparisons. We recorded 10-day-old pup ultrasonic vocalizations in a sound-attenuating chamber, as described. Tests were conducted between 900 and 1400 h and lasted for 15 s. Drugs were administered after baseline value collection (15 s) and pups were tested again 30 min after drug administration. Data were expressed as percent change from baseline and analyzed by overall one-way ANOVA followed by Tukey’s test for individual between-group comparisons.

Competing interests statement
The authors declare competing financial interests: see the website http://www.nature.com/naturemedicine for details.

Acknowledgments
We thank A. Giulfrida and M. Solbrig for critical reading of the manuscript; B. Ferrer and F. Rodríguez de Fonseca for help with initial experiments; and J. Yoo and C. Park for technical assistance. The assistance of the Centro Interfacoltà Parma and Urbino. D.P. is a Staglin Music Festival Investigator of the National Institutes of Health and A. Giulfrida and M. Solbrig for critical reading of the manuscript.