A catalytically silent FAAH-1 variant drives anandamide transport in neurons

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The endocannabinoid anandamide is removed from the synaptic space by a selective transport system, expressed in neurons and astrocytes, that remains molecularly uncharacterized. Here we describe a partly cytosolic variant of the intracellular anandamide-degrading enzyme fatty acid amide hydrolase-1 (FAAH-1), termed FAAH-like anandamide transporter (FLAT), that lacked amidase activity but bound anandamide with low micromolar affinity and facilitated its translocation into cells. Known anandamide transport inhibitors, such as AM404 and OMDM-1, blocked these effects. We also identified a competitive antagonist of the interaction of anandamide with FLAT, the phthalazine derivative ARN272, that prevented anandamide internalization in vitro, interrupted anandamide deactivation in vivo and exerted profound analgesic effects in rodent models of nociceptive and inflammatory pain, which were mediated by CB1 cannabinoid receptors. The results identify FLAT as a critical molecular component of anandamide transport in neural cells and a potential target for therapeutic drugs.

Brain cells release a variety of lipid mediators, which act in close proximity to their site of production to modulate synaptic plasticity and neural development1. The reliability of this highly localized form of neural communication depends on the existence of deactivation mechanisms that ensure the rapid termination of lipid-mediated signaling, but few such mechanisms have been discovered so far. Anandamide is an arachidonic acid derivative that regulates ion-channel activity and neurotransmitter release by engaging CB1 cannabinoid receptors on axon terminals2. There is evidence that the intensity and duration of anandamide signaling are controlled by a two-step elimination process in which the substance is first internalized by neurons and astrocytes3–5 and then hydrolyzed by the intracellular membrane-bound amidases FAAH-1 and FAAH-2 (refs. 6–8). Removal of anandamide from the extracellular space shows several identifying features of a carrier-mediated facilitated diffusion process1,9,10: (i) it is saturable and has low micromolar affinity for anandamide (apparent Michaelis constant $K_M$, 1.2 µM in rat cortical neurons)3; (ii) it preferentially recognizes anandamide over similar molecules, including the non-cannabinoid FAAH substrates oleoylthanolamide (OEA) and palmitoylethanolamide (PEA)3,11; (iii) it is inhibited in a competitive and stereoselective manner by substrate mimics10; and (iv) it does not require cellular energy3,4. Inhibitors of anandamide transport, which include the compounds AM404 and OMDM-1 (ref. 10), increase amounts of this endocannabinoid substance in vivo and produce a spectrum of CB1-mediated responses that only partially overlap with those elicited by FAAH blockade, presumably owing to the different kinetic properties and substrate preferences of the two deactivation mechanisms10. These data indicate that carrier-mediated transport may be important for terminating the actions of anandamide and might represent a potential drug target10. Nevertheless, the molecular entity (or entities) involved in anandamide translocation is still unknown, and the mechanistic bases of this process remain controversial5,12. Here, we identify a partly cytosolic variant of FAAH-1, termed FLAT, that lacks amidase activity but binds anandamide with low micromolar affinity and confers anandamide transport on cells that are engineered to express it. AM404 and other anandamide transport inhibitors suppress these effects. Moreover, we disclose a small-molecule competitive inhibitor of the interaction of anandamide with FLAT, the compound ARN272, and show that this agent suppresses anandamide translocation in vitro and interrupts anandamide deactivation in vivo.

RESULTS

FLAT is an intracellular anandamide-binding protein

We isolated total RNA from brain and other rat tissues, and amplified products of the Faah gene using reverse transcriptase polymerase chain reaction (RT-PCR). One of the cDNA products obtained was identical to Faah except that it lacked a 204-base-pair segment encoding amino acid residues 9–76 (Fig. 1a, Supplementary Fig. 1a–c). RNase protection assays and Southern blot analyses of reverse transcriptase–generated cDNA confirmed the occurrence of FLAT mRNA in rat brain and liver tissue (Supplementary Fig. 1d,e). Quantitative RT-PCR measurements showed that FLAT was unevenly transcribed in the rat brain, with highest

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amounts in neocortex and hippocampus and lowest in brainstem and hypothalamus (Supplementary Fig. 1f). Detectable FLAT mRNA was also found in rat primary astrocytes in cultures, rat neuroblastoma cells and human astrocytoma cells (Supplementary Fig. 1c), which were previously shown to have anandamide transport activity3,11,13. An antibody raised against the C terminus of FAAH-1 identified in brain cytosolic and membrane fractions obtained from wild-type mice, but not in those obtained from FAAH-1-deficient mice, a band with an apparent molecular weight of 56 kDa, which is consistent with the calculated molecular weight of FLAT (56,008 Da) (Supplementary Fig. 1g). This suggests that FLAT might be a product of the Faah gene generated by alternative splicing at noncanonical sites 14. The predicted structure of FLAT lacks most of FAAH-1’s α1 helix, which spans the lipid bilayer of intracellular membranes, and the entire α2 helix, which flanks the globular body of the protein exposed to the cytosol (Fig. 1b)15.

When expressed in HEK-293 cells, FLAT showed no detectable amidase activity toward anandamide or OEA (Supplementary Fig. 2a,b), suggesting that the protein is catalytically deficient. Computational studies identified two factors that might contribute to this loss of activity: (i) increased flexibility of regions proximal to the missing α1 and α2 helices, such as the ‘α2-interacting loop’ (Fig. 1b), could facilitate access of water to the catalytic site buried in the enzyme’s hydrophobic core (Supplementary Fig. 3)7; and (ii) deletion of the α2 helix, which carries a positively charged surface, could lower the electrostatic potential in the region surrounding the catalytic triad component Lys74 (corresponding to Lys142 in FAAH-1)7 (Supplementary Fig. 4). Both factors are expected to impair amidase activity toward anandamide or OEA (Supplementary Fig. 4). We detected appreciable amounts of FLAT in cytosol fractions prepared from mouse brain (Supplementary Fig. 1g) or transfected HEK-293 cells (Fig. 2c) and found that the protein could be readily detected from HEK-293 cell membranes by incubation with sodium carbonate (0.1 M) (Supplementary Fig. 6)19. These properties, along with the observation that AM404 and OMDM-1 antagonize the binding of [3H]anandamide to FLAT (Fig. 2b), likely because the productive interaction of this compound with the Ser241 nucleophile of FAAH-1 requires a fully functional catalytic triad18. Collectively, the experiments described above indicate that FLAT lacks amidase activity but binds anandamide with low micromolar affinity.

FLAT facilitates anandamide transport

We detected appreciable amounts of FLAT in cytosol fractions prepared from mouse brain (Supplementary Fig. 1g) or transfected HEK-293 cells (Fig. 2c) and found that the protein could be readily detached from HEK-293 cell membranes by incubation with sodium carbonate (0.1 M) (Supplementary Fig. 6)19. These properties, along with the observation that AM404 and OMDM-1 antagonize the binding of [3H]anandamide to FLAT (Fig. 2b), suggested a role in anandamide translocation. Such a role was further implied by the reduced [3H]anandamide accumulation observed in cultures of brain neurons obtained from Faah-/- mice (Supplementary Fig. 7)20,21, which lack both FAAH-1 and FLAT (Supplementary Fig. 1g). We examined therefore whether heterologous expression of FLAT might increase anandamide transport in HEK-293 cells. We incubated control and FLAT-expressing cells for 5 min at 37 °C in a buffer containing [3H]anandamide and measured cell-associated radioactivity after removal of excess tracer3,11. Compared to controls, FLAT-expressing cells had significantly higher [3H]anandamide...
ARN272 is a competitive FLAT inhibitor. (a) Effects of ARN272 on [3H]anandamide binding to FLAT-GST. Inset: chemical structure of ARN272. (b) Effects of ARN272 (concentrations in μM) on [3H]anandamide accumulation in FLAT-expressing HEK-293 cells (black bars); white bar, vector-transfected cells. (c) Effects of vehicle (open bar), ARN272 and AM404 (closed bars) on [3H]anandamide accumulation in rat cortical neurons in cultures. (d) Effects of ARN272 and URB597 on FAAH activity in rat brain membranes. (e,f) Effects of ARN272 (1 mg kg−1, i.p.) on plasma concentrations of anandamide (e) or OEA, PEA and 2-AG (f) in mice. Results are expressed as mean ± s.e.m. of three to seven experiments. ***P < 0.001 versus vehicle. #P < 0.05 and ##P < 0.01 versus vehicle, one-way ANOVA followed by Dunnett’s test.

In addition to internalizing anandamide, the anandamide transport system may also facilitate the release of this lipid mediator from cells. To test whether FLAT contributes to this process, we overexpressed the protein in mouse Neuro-2a cells and measured, by liquid chromatography/mass spectrometry, endogenously produced anandamide in the incubation medium. Consistent with a role in anandamide release, FLAT overexpression was accompanied by a substantial elevation in extracellular anandamide, but not 2-AG or OEA (Supplementary Fig. 8). The results suggest that FLAT facilitates anandamide translocation through a mechanism that is selective for anandamide, independent of amidase activity and prevented by known inhibitors of anandamide transport.

Figure 2 FLAT binds to anandamide and facilitates its transport into cells. (a) Specific binding of [3H]anandamide to rat FLAT–glutathione-S-transferase (GST) (filled squares) or GST alone (open squares). Inset: Scatchard transformation (bound – (bound/free), in nmol) of binding data. (b) AM404 (open squares) and OMDM-1 (filled squares) antagonize [3H]anandamide binding to FLAT-GST, whereas URB597 (open circles) has no effect. (c) Cytosolic fractions of FLAT-expressing HEK-293 cells (right lane) contain detectable amounts of FLAT (arrow); a corresponding fraction from FAAH-1-expressing cells is shown for comparison (left lane). Bottom band, β-actin. (d) [3H]Anandamide accumulation in control cells (vector-transfected, white bar) or FLAT-expressing HEK-293 cells (black bars) incubated with vehicle (0.01% dimethylsulfoxide), AM404 or nonradioactive anandamide (concentrations in μM). (e) The anandamide transport inhibitors OMDM-1, UCM707 and VDM11 suppress [3H]anandamide accumulation in FLAT-expressing HEK-293 cells. This process is not affected by URB597 or mutation of catalytic Ser242 (gray bar). (f) Accumulation of 3H-labeled lipids in control (white bars) or FLAT-expressing HEK-293 cells (black bars). Anandamide, AEA; arachidonic acid, AA. Results are expressed as mean ± s.e.m. of three to seven experiments. ***P < 0.01 versus vector-transfected cells, Student’s t-test; #P < 0.05, ##P < 0.01 and ###P < 0.001 versus vehicle, one-way analysis of variance (ANOVA) followed by Dunnett’s test.

accumulation, which (i) was prevented by AM404 (IC50 = 4 μM) and other transport inhibitors (OMDM-1, UCM707, VDM11), as well as by nonradioactive anandamide (100 μM) (Fig. 2d,e); and (ii) was selective for anandamide over four structurally related lipids: the FAAH substrates [3H]OEA and [3H]PEA, the eicosanoid precursor [3H]arachidonic acid and the endocannabinoid fatty acyl ester [3H]2-arachidonoyl-sn-glycerol (2-AG) (Fig. 2f). [3H]Anandamide accumulation in FLAT-expressing HEK-293 cells may not be attributed to passive diffusion driven by FAAH-mediated hydrolysis22, because the very low amidase activity present in native HEK-293 cells was not increased by FLAT expression (Supplementary Fig. 2a,b). Moreover, treatment with a maximally active concentration of the FAAH inhibitor URB597 (1 μM) or mutation of Ser173 (corresponding to the nucophile Ser241 in FAAH-1) did not affect [3H]anandamide uptake by FLAT-expressing HEK-293 cells (Fig. 2e). URB597 did not affect [3H]anandamide uptake at any of the concentration tested (up to 10 μM: 103 ± 5% of vehicle control (mean ± s.e.m.)).
Intraplantar injection of formalin (5%, 20 μl) elicited two temporally distinct phases of nocifensive behavior in mice: phase 1 (0–5 min, white bars) and phase 2 (5–45 min, black bars). (a) ARN272 (doses in mg kg⁻¹, i.p.) decreased nocifensive behavior in both phases. (b) The CB₁ antagonist AM251 (1 mg kg⁻¹, i.p.) abolished the antinociceptive effects of ARN272, whereas the CB₂ antagonist AM630 and the TRPV1 antagonist AMG9810 did not. (c–f) Intraplantar injection of carrageenan (car) elicited a local inflammatory response in mice. ARN272 (mg kg⁻¹, i.p.) decreased (c) thermal hyperalgesia (withdrawal latency), and (d) edema (volume). The CB₁ antagonist AM251 (1 mg kg⁻¹, i.p.) suppressed the effects of ARN272 on (e) thermal hyperalgesia and (f) edema. Results are expressed as the mean ± s.e.m. of six mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001 versus vehicle-injected controls, two-way ANOVA followed by Bonferroni’s test.

Figure 4. ARN272 produces CB₁-dependent antinociception in mice. Intraplantar injection of formalin (5%, 20 μl) elicited two temporally distinct phases of nocifensive behavior in mice: phase 1 (0–5 min, white bars) and phase 2 (5–45 min, black bars). (a) ARN272 (doses in mg kg⁻¹, i.p.) decreased nocifensive behavior in both phases. (b) The CB₁ antagonist AM251 (1 mg kg⁻¹, i.p.) abolished the antinociceptive effects of ARN272, whereas the CB₂ antagonist AM630 and the TRPV1 antagonist AMG9810 did not. (c–f) Intraplantar injection of carrageenan (car) elicited a local inflammatory response in mice. ARN272 (mg kg⁻¹, i.p.) decreased (c) thermal hyperalgesia (withdrawal latency), and (d) edema (volume). The CB₁ antagonist AM251 (1 mg kg⁻¹, i.p.) suppressed the effects of ARN272 on (e) thermal hyperalgesia and (f) edema. Results are expressed as the mean ± s.e.m. of six mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001 versus vehicle-injected controls, two-way ANOVA followed by Bonferroni’s test.

DISCUSSION

The functional properties of FLAT suggest that this protein is a key molecular component of the anandamide transport system in neural cells and is a potential target for therapeutic drugs. The findings presented here indicate that FLAT selectively binds to and internalizes anandamide and that several known inhibitors of anandamide translocation—AM404, OMDM-1, UCM707 and VDM11 (ref. 10)—interfere with these properties. Moreover, our results show that ARN272, a small-molecule inhibitor of the interaction of anandamide with FLAT, suppresses anandamide accumulation by rat brain
neurons in vitro and reproduces two key effects of transport blockade in vivo: elevation of plasma anandamide, and analgesia in models of nociceptive and inflammatory pain. Consistent with these data and previous reports, deletion of the Faah gene substantially reduced anandamide transport in mouse cortical neurons, whereas acute pharmacological blockade of FAAH activity did not do so.

Although implying that FLAT is important in anandamide transport, our findings do not rule out the possibility that more components of the endocannabinoid transport system remain to be discovered. In this context, we note that FLAT expression did not confer [3H]2-AG or [3H]OEA transport on HEK-293 cells, and administration of the FLAT inhibitor ARN272 did not increase plasma 2-AG or OEA in mice, which indicates that the translocation of these lipid mediators may be independent of FLAT. Because of its ability to inhibit anandamide deactivation selectively, ARN272 may be useful to differentiate the functions of anandamide from those of other lipid amides that are substrates for FAAH (for example, OEA and PEA).

Multicellular organisms use protein carriers to coordinate the traffic of functionally important lipids and target these biomolecules toward specific cells and subcellular compartments. Two main types of lipid-carrier proteins are used for this task: integral membrane transporters, such as CD36 (ref. 30) and PGT31, and lipid chaperones, such as aP2 and mal1 (fatty acid-binding proteins 4 and 5, respectively)32. For example, membrane-bound CD36 in small intestinal enterocytes captures dietary oleic acid and directs it toward the intracellular bioavailability of OEA, an important gut hormone. However, cytosolic aP2 in adipocytes encapsulates fatty acids derived from the circulation and partitions them toward appropriate cellular sites for storage or oxidative metabolism. Our experiments suggest that, similarly to a lipid chaperone, FLAT might function by desorbing anandamide from the cell membrane and delivering it to intracellular organelles where FAAH-1 is located (Supplementary Fig. 15). It is also possible, though remains to be fully tested, that FLAT might contribute to anandamide release by facilitating the intracellular transfer of this lipophilic molecule from its as-yet-unknown site of biosynthesis to the cell membrane.

Despite its similarities with other lipid chaperones, FLAT appears to be functionally distinct in at least two ways. First, its substrate preference and sensitivity to pharmaceutical agents distinguish it from other carriers for lipophilic ligands, such as serum albumin and fatty acid–binding proteins, which are known to sequester anandamide in a lipid microdomain that is not as well defined. Second, our studies suggest that the capacity of FLAT to ligate anandamide may be based on structural modifications that silence the amidase activity of FAAH-1 without compromising its anandamide-binding function. This mechanism provides an elegant example of phylogenetic parsimony and raises the possibility that other lipid transporters might have evolved following similar principles.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

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AUTHOR CONTRIBUTIONS

J.F. conducted the molecular biological, biochemical and pharmacological experiments in vitro and analyzed data. G.B. conducted the ligand screening campaign. G.B., W.R., M. Massetti, A.L. and A.C. performed the computational studies. T.B. provided chemical expertise. O.S., R.B. and A.R. conducted the pharmacological experiments in vivo and analyzed data. A.G. measured lipid levels in vivo and analyzed data. A.A. and G.G. conducted analytical studies on purified recombinant FLAT. D.P. conceived and designed the experiments, oversaw the project and wrote the manuscript with assistance from J.F., G.B., W.R., M. Mor, A.L. and A.C.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/natureneuroscience/.

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ONLINE METHODS

FLAT model. The α2 helix (residues 37 to 68) was removed from the crystal structure of FAAH-1-ΔTM (PDB 1MT5) and positions 69 to 75 modeled de novo. The cocrystallized ligand and water molecules were removed. Hydrogen atoms and missing heavy atoms were added. Side chains of residues 69–75, zero occupancy side chains and polar hydrogen atoms were assigned the lowest energy conformations. Tautomeric states of histidines and the positions of asparagine and glutamine side chain amido groups were optimized to improve the hydrogen bond pattern.

Monte Carlo simulations. Conformational analysis of the α2-interacting loop was performed using the Metropolis biased probability Monte Carlo (BPMC) method as implemented in ICM3.7 (ref. 40). The initial temperature was set at 600 K. A total of 5 × 10^7 energy evaluations were carried out. The variables sampled during the simulation were the backbone and side chain torsional angles of the loop amino acids. The side chain torsional variables of all the amino acids with at least one heavy atom within 2 Å from the loop were minimized together with the sampled variables before acceptance or rejection according to the Metropolis criterion.

Molecular dynamics simulations. Molecular dynamics simulations were conducted using the NAMD 2.6 (ref. 41) software and the Amber95SB force field42. FLAT and FAAH-1-ΔTM were solvated in explicit solvent. The dimensions of the simulation box were chosen so to keep a margin of at least 8 Å from the solute in each coordinate direction, and the electro-neutrality of the cell was reached by adding counter-ions. The simulations were performed in the isobaric-isothermal statistical ensemble with periodic boundary conditions. Langevin dynamics was undertaken at the temperature of 300 K with a frictional coefficient of 5 ps^-1 at the target pressure of 1 atm. A uniform time-stepping of 2 fs was used. Bonds involving hydrogen atoms were restrained to their reference value. Short-range non-bonded interactions were calculated using a distance cutoff of 10 Å. Long-range electrostatic interactions were considered using the particle mesh Ewald method. The system was thermalized during 150 ps of molecular dynamics while smoothly releasing restraints initially applied on the protein heavy atoms. The system was then allowed to evolve for a total simulated time of 100 ns.

Electrostatic simulations. The Poisson-Boltzmann equation was solved over a system in which FLAT was modeled as a polarizable solute and the solvent was modeled as a high-dielectric continuum plus an ionic strength of 0.145 M. Electrostatic potential values generated by representative structures of FLAT and FAAH-1-ΔTM at the ζ nitrogen site of Lys142 were calculated and compared using DelPhi software43. The simulations suggested that the α2 helix brings a positive electrostatic contribution to the lysine site. This contribution is the sum of the mere helix dipolar potential and the overall net charge potential generated by titratable residues borne by the α2 helix. In FLAT, this effect is only partially compensated by the reaction field generated by the solvent that replaces the α2 helix. The pKₐ shift between the two forms was estimated quantitatively by performing electrostatic calculations according to a conceptual thermodynamic cycle:

$$\Delta pK_a = pK_a^{\text{FLAT}} - pK_a^{\text{ΔTM}} = \left([G_{\text{FLAT}}^0 - G_{\text{FLAT}}^+ - (G_{\text{ΔTM}}^0 - G_{\text{ΔTM}}^+)]/2.303RT\right)$$

where ΔTM represents FAAH-1-ΔTM. This calculation provided a positive average pKₐ shift of about 0.8 pKₐ units, pointing to an increased probability of having a protonated catalytic lysine in FLAT.

Virtual ligand screening. Database selection and pre-filtering. We retrieved 4,325,889 unique structures for virtual ligand screening (VLS) from the Molcart database v1.9.6 (Molsoft). First, compounds were filtered using the physicochemical profile expected of a potential transport inhibitor: molecular weight 300–400 Da, log (partition coefficient P) 4–8, hydrogen bond donors 0–3, hydrogen bond acceptors 2–4, polar surface area 20–80 Å², molecular volume 340–485 Å³, ring terms 3–7. This profile was defined using five templates: (i) anandamide, (ii) AM404, (iii) a reversible FAAH thiohydantoin inhibitor (compound 123 in ref. 44), (iv) OL-92 and (v) the leaving group of URB597. The first filtering step retained 288,307 compounds that matched the sought physicochemical profile. Then, to avoid a bias toward known scaffolds, molecules within a Tanimoto distance between 0 and 0.6 (with 0 indicating that two molecules share an identical fingerprint generated according to the Daylight algorithm (Daylight Chemical Information Systems) from any of the five template compounds were discarded. At the end of the prefiltering steps, 122,095 unique molecules were selected.

Docking procedure. The boundaries of the binding pocket were calculated using the Pocketsome Gaussian Convolution algorithm as implemented in ICM3.7. The three-dimensional models of 122,095 molecules were automatically built and docked at the FLAT binding site according to the ICM3.7 standard docking procedure. This step identified 4,601 compounds that matched the energy criterion of −32 arbitrary score units.

Cluster analysis and visual inspection. The chosen compounds were submitted to an unweighted pair group method with arithmetic mean (UPGMA) chemical clustering procedure. The functional partition was selected at a threshold value of 0.5 Tanimoto distances. This returned 56 unique clusters. Depending on the cluster cardinality, one to three representatives were selected, providing 124 potential FLAT binders. After visual inspection, 53 compounds were selected and 46 that were available from commercial vendors were subjected to testing in vitro.

Neuronal cultures. Primary cultures of mouse and rat cortical neurons were prepared as described in reference 45.

FLAT cloning and expression. Rat FLAT mRNA was amplified by PCR using the following primers: forward, 5′-ACCATGTTGCTCTAGGAAGTTGAGGACCC-3′; reverse, 5′-TCCAGCATTGCTGTTTGTGAGGTGTTG-3′. A rat FLAT amplicon was subcloned into the pcDNA vector using the TOPO TA Cloning kit (Invitrogen) and splicing was confirmed by sequencing. Amiplicons of rat FAAH-1, FLAT and mutated FLAT-S170G were cloned into a pcDNA3.1 expression vector (Invitrogen) under the control of a human cytomegalovirus promoter. HEK-293 cells were cultured in DMEM supplemented with 10% (vol/vol) FBS and transfected with Lipofectamine 2000 (10 μl, Invitrogen) containing 1 μg of plasmids. Culture media were replaced 18 h after transfection with DMEM containing G418 (0.2 mg ml⁻¹, Calbiochem). After 4 weeks in culture, surviving clones were isolated and analyzed by western blot to select cell lines stably expressing the transgenes. Ser173 in rat FLAT was mutated to glycine using the GeneTailor mutagenesis system (Invitrogen).

RT-PCR. RNA was extracted with TRIzol (Invitrogen), and cDNA was synthesized from 1 μg of total RNA. PCR was conducted using the following primers: forward, 5′-CACATGTTGCTCTAGGAAGTTGAGGACCC-3′; reverse, 5′-TCCAGCATTGCTGTTTGTGAGGTGTTG-3′. mRNA expression was determined using glyceraldehyde 3-phosphate dehydrogenase as standard.

RNase protection assays. Probes comprising nucleotides 20–309 encoding FAAH-1 and nucleotides 20–220 encoding FLAT were generated by PCR followed by subcloning into pcDNA vectors. The constructs were linearized by digestion with BamHI and used as templates for in vitro transcription incorporated with [32P]-cTP (MP Biomedicals) using RNA polymerase SP6 (Roche). RNase protection assays were performed with an RPA III kit (Applied Biosystems).

Southern blot analyses. Total RNA was extracted from tissues with TRIzol. cDNA was synthesized from 20 μg of total RNA using SuperscriptII RNase H−reverse transcriptase (Invitrogen). cDNA was digested with Bpl and separated by electrophoresis at 30 V overnight. Southern blots were performed using
a standard protocol with a 5'-terminal probe (237 nt, from nt 41–277) or a 3'-terminal probe (409 nt, from nt 601–1010) for FAAH-1.

**Western blots.** Protein (20 µg) were subjected to electrophoresis on 4–15% SDS-PAGE gels and transferred onto Immobilon membranes (Millipore). Western blots were run using antibodies to the V protein of simian virus 5 (V5) (1:3,000, Invitrogen), FAAH-1 (1:500, Abbiotec) and β-actin (1:10,000, Calbiochem). Bands were visualized with an Electrochemiluminescence Plus kit (Amersham). Quantitative analyses were performed using US National Institutes of Health Image software, using β-actin as an internal standard.

**FLAT binding assays.** A pGEX-rFLAT plasmid containing coding sequences for fused rat FLAT and GST was constructed in a pGEX-4T vector (Amersham) and digested with BamHI. The GST-FLAT fusion protein was generated in *Escherichia coli* (BL21 strain, Novagen). After induction, bacteria were grown at 27 °C for 10 h and the protein purified using glutathione-Sepharose beads (Amersham). Purified GST-FLAT was incubated at 25 °C for 2 h in HEPES buffer (50 mM, pH 7.0) containing KCl (50 mM), EDTA (5 mM), dithiothreitol (10 mM) and [3H]anandamide (100 nM, 15 Ci mmol⁻¹). Free [3H]anandamide was separated on a Sephadex G-25 spin column (Amersham) and radioactivity in the bound fraction was measured. Nonspecific binding was determined in the presence of nonradioactive anandamide (10 µM).

**Anandamide translocation.** HEK-293 cells or cortical neurons in cultures were incubated for 5 min at 37 °C in Tris–Krebs buffer (125 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, 1.2 mM NaHPO₄, 2.5 mM CaCl₂), pH 7.5, containing [3H]anandamide or other 3H-labeled lipids (each at 200 nM; 10,000 d.p.m., specific activity 20 Ci mmol⁻¹). Cells were rinsed with Tris–Krebs and scrapped into NaOH (0.2 M), and radioactivity was measured. Test compounds were added to the cultures 10 min before [3H]anandamide.

**Anandamide release.** Neuro-2A cells (1 × 10⁶) were cultured overnight in KCl, 25 mM NaHCO₃, 1.2 mM NaHPO₄, 2.5 mM CaCl₂), pH 7.5, containing [3H]anandamide or other 3H-labeled lipids (each at 200 nM; 10,000 d.p.m., specific activity 20 Ci mmol⁻¹). Supernatants were collected, and lipids were extracted with methanol/chloroform (1:2, vol/vol) containing [3H]OEA, [3H]anandamide and [3H]2-AG (100 pmol). After centrifugation at 1,500g for 15 min, organic phases were collected and dried. Lipids were reconstituted in methanol (0.1 ml) and quantified by LC/MS⁴⁷.

**Behavioral tests.** We used male CD1 mice weighing 25–30 g (Charles River). The mice had free access to food and water and were maintained under a 12 h light/dark cycle at controlled temperature and relative humidity. Behavioral experiments were performed in accordance with the Ethical Guidelines of the International Association for the Study of Pain and approved by Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with European Economic Community regulations (O.J. of E.C. L 358/1 12/18/1986). We injected formalin (5% in sterile saline, 10 µl) into the plantar surface of the left hind paw and monitored nocifensive behavior (licking and biting of the injected paw) as described⁴⁶. Edema was elicited by injecting λ-carrageenan (1% wt/vol, 20 µl) into the left hindpaw of mice. Edema was measured with a plethysmometer (Ugo Basile). Thermal hyperalgesia was assessed as described⁴⁷. Vehicle or ARN272 were administered by i.p. (0.01–3 mg kg⁻¹) or intracerebroventricular (0.0–3 µg per CD1 mouse) injection immediately before formalin or carrageenan. Antagonists were injected (1 mg kg⁻¹, i.p.) 30 min before formalin or carrageenan.

**Lipid measurements ex vivo.** CD1 mice received i.p. injections of vehicle (5% polyethylene glycol 400, 5% Tween-80 in saline, 5 ml kg⁻¹) or ARN272 (1 mg kg⁻¹) and were killed 1–2 h later under isoflurane anesthesia. Blood was collected through a left cardiac puncture and centrifuged at 3000g for 30 min. Plasma (0.2 ml) was incubated with 1 ml cold acetone, centrifuged at 1,500g for 15 min at 4 °C and suspended in 50% methanol (2 ml) containing [3H]anandamide, [3H]OEA, [3H]PEA and [3H]2-AG. Lipids were extracted with chloroform (2 ml) and organic phases were collected and dried under nitrogen. Lipids were reconstituted in methanol (0.1 ml) and measured by LC/MS.

**Statistical analyses.** Results are expressed as the mean ± s.e.m. of n separate experiments. The significance of differences between groups was evaluated by one-way analysis of variance (ANOVA) followed by a Dunnett's test for multiple comparisons or a Student's t-test. For behavioral experiments, differences were evaluated by two-way ANOVA followed by a Bonferroni's test. Analyses were conducted using the GraphPad Prism software, and differences were considered significant if P < 0.05.