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

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Abstract

The endocannabinoid anandamide is removed from the synaptic space by a selective transport system, expressed in neurons and astrocytes, which 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), which lacks amidase activity but binds anandamide with low micromolar affinity and facilitates its translocation into cells. Known anandamide transport inhibitors, such as AM404 and OMDM-1, block these effects. Additionally, we identify a competitive antagonist of the interaction of anandamide with FLAT, the phthalazine derivative ARN272, which prevents anandamide internalization in vitro, interrupts anandamide deactivation in vivo, and exerts profound analgesic effects in rodent models of nociceptive and inflammatory pain, which are mediated by CB_1 cannabinoid receptors. The results identify FLAT as a critical molecular component of anandamide transport in neural cells and a potential target for therapeutic drugs.

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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 virtual ligand screening campaign. G.B., W.R., M.M., 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.M., A.L., and A.C.

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INTRODUCTION

Brain cells release a variety of lipid mediators, which act in close proximity of their site of production to modulate synaptic plasticity and neural development[1]. 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 CB_1 cannabinoid receptors on axon terminals[2]. 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 astrocytes[3–5] and then hydrolyzed by the intracellular membrane-bound amidases, FAAH-1 and FAAH-2[6–8]. Removal of anandamide from the extracellular space exhibits several identifying features of a carrier-mediated facilitated diffusion process[4,9,10]: (*i*) it is saturable and displays 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 oleoylethanolamide (OEA) and palmitoylethanolamide (PEA)[3,11]; (*iii*) it is inhibited in a competitive and stereoselective manner by substrate mimics[10]; and (*iv*) it does not require cellular energy[3,4]. Inhibitors of anandamide transport - which include the compounds AM404 and OMDM-1[10] - increase the levels of this endocannabinoid substance in vivo and produce a spectrum of CB_1-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 mechanisms[10]. These data indicate that carrier-mediated transport may play an important role in terminating the actions of anandamide and might represent a potential drug target[10]. Nevertheless, the molecular entity (or entities) involved in anandamide translocation is still unknown and the mechanistic bases of this process remain controversial[5,12]. Here, we identify a partly cytosolic variant of FAAH-1, termed FLAT, which lacks amidase activity but binds anandamide with low micromolar affinity and confers anandamide transport to 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-1* gene using reverse-transcriptase polymerase chain reaction (RT-PCR). One of the complementary DNA products obtained was identical to *faah-1* except that it lacked a 2 0 4 base-pair segment encoding for amino-acid residues 9–76 (Fig. 1a, Supplementary Fig. 1a–c). Ribonuclease protection assays and Southern blot analyses of RT-generated cDNA confirmed the normal occurrence of FLAT mRNA in rat brain and liver tissue (Supplementary Fig. 1d,e). Quantitative RT-PCR measurements showed that FLAT is unevenly transcribed in the rat brain, with highest levels in neocortex and hippocampus and

lowest levels in brainstem and hypothalamus (Supplementary Fig. 1f). Detectable levels of FLAT mRNA were also found in rat primary astrocytes in cultures, rat neuroblastoma cells, and human astrocytoma cells (Supplementary Fig. 1c), which were previously shown to express anandamide transport[3,11,13]. 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-1* gene generated by alternative splicing at non-canonical 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 Hek293 cells, FLAT displayed no detectable amidase activity toward anandamide or OEA (Supplementary Fig. 2a,b), suggesting that the protein is catalytically defective. 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 inside 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 Lys^{74} (corresponding to Lys^{142} in FAAH-1)[7] (Supplementary Fig. 4). Both factors are expected to impair amidase activity by interrupting the proton transfer from Ser^{173} (corresponding to catalytic Ser^{241} in FAAH-1) to neutral Lys^{74}. Though critical for the amidase activity of FAAH-1, Lys^{142} does not influence the ability of this enzyme to cleave ester substrates[16]. Consistent with those data and the model proposed here, we found that recombinant FLAT effectively hydrolyzes the fatty acyl ester, 2-oleoyl-*sn*-glycerol (Supplementary Fig. 2c).

Like FAAH-1, recombinant purified FLAT forms homodimers in aqueous solution (Supplementary Fig. 5). To test whether FLAT also retains the ability to ligate anandamide, we expressed the protein fused with glutathione-*S*-transferase (GST) in *E. coli* and purified it by affinity chromatography. Saturation binding studies showed that [^{3}H]-anandamide associates with FLAT-GST (dissociation constant, Kd=2 μM), but not with GST alone (Fig. 2a). The binding of [^{3}H]-anandamide to FLAT is displaced by the anandamide transport inhibitors AM404 and OMDM-1 (Fig. 2b), with median inhibitory concentrations similar to those required for the inhibition of neuronal [^{3}H]-anandamide internalization (IC_{50}: AM404, 5.3 μM; OMDM-1, 4.8 μM)[10,17]. By contrast, the covalent FAAH inhibitor URB597 had no such effect (Fig. 2b), likely because the productive interaction of this compound with the Ser^{241} nucleophile of FAAH-1 requires a fully functional catalytic triad[18]. Collectively, the experiments described above indicate that FLAT lacks amidase activity, but binds anandamide with low micromolar affinity.

A role for FLAT in anandamide transport

We detected significant amounts of FLAT in cytosol fractions prepared from mouse brain (Supplementary Fig. 1g) or transfected Hek293 cells (Fig. 2c), and found that the protein can

be readily detached from Hek293 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 [^{3}H]-anandamide to FLAT (Fig. 2b), are suggestive of a role in anandamide translocation. Such a role was further implied by the reduced [^{3}H]-anandamide accumulation observed in cultures of brain neurons obtained from *faah-1*$^{-/-}$ mice (Supplementary Fig. 9)[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 Hek293 cells. We incubated control and FLAT-expressing cells for 5 min at 37°C in a buffer containing [^{3}H]-anandamide, and measured cell-associated radioactivity after removal of excess tracer[3,11]. Compared to controls, FLAT-expressing cells displayed a significantly higher level of [H^3]-anandamide accumulation, which (*i*) was prevented by AM404 (IC_{50} = 4 μM) and other transport inhibitors (OMDM-1, VDM11, UCM707), as well as by non-radioactive anandamide (100 μM) (Fig. 2D,E); and (*ii*) was selective for anandamide compared to four structurally related lipids: the FAAH substrates [^{3}H]-OEA and [^{3}H]-PEA, the eicosanoid precursor [^{3}H]-arachidonic acid, and the endocannabinoid fatty acyl ester [^{3}H]-2-arachidonoyl-*sn*-glycerol (2-AG) (Fig. 2f). [^{3}H]-Anandamide accumulation in FLAT-expressing Hek293 cells may not be attributed to passive diffusion driven by FAAH-mediated hydrolysis[22], because the very low amidase activity present in native Hek293 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 Ser^{173} (corresponding to the nucleophile Ser^{241} in FAAH-1) did not affect [^{3}H]-anandamide uptake by FLAT-expressing Hek293 cells (Fig. 2e). URB597 did not affect [^{3}H]-anandamide uptake at any of the concentration tested (up to 10 μM: 103±5% of vehicle control).

In addition to internalizing anandamide, the anandamide transport system may also facilitate the release of this lipid mediator from cells[4,23]. To test whether FLAT contributes to this process, we over-expressed the protein in mouse Neuro-2a cells and measured, by liquid chromatography/mass spectrometry, the levels of endogenously produced anandamide in the incubation medium. Consistent with a role in anandamide release, FLAT over-expression was accompanied by a significant elevation in the extracellular levels of anandamide, but not 2-AG or OEA (Supplementary Fig. 7). 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.

Discovery of a competitive FLAT inhibitor

To further investigate the functions of FLAT, and differentiate them from those of FAAH-1, we searched for small drug-like molecules that might selectively interfere with FLAT's ability to sequester anandamide. We subjected a virtual 4.3 million compound library to a ligand-screening campaign structured in multiple steps of progressively increasing stringency (Supplementary Fig. 8). The campaign returned a set of 46 structurally diverse compounds, which were tested for their interaction with FLAT in vitro. One of them, the substituted phthalazine ARN272 (Fig. 3a), competitively antagonized [^{3}H]-anandamide binding to purified FLAT (IC_{50}=1.8 μM; Fig. 3a) and inhibited [^{3}H]-anandamide

accumulation in both FLAT-expressing Hek293 cells (IC_{50}≈3 μM; Fig. 3b) and primary cultures of cortical neurons prepared from rats (Fig. 3c) or wild-type mice (Supplementary Fig. 9). By contrast, ARN272 exerted no significant effect on the residual [^{3}H]-anandamide accumulation observed in cortical neurons of *faah-1*$^{-/-}$ mice (Supplementary Fig. 9).

FLAT inhibition by ARN272 appeared to be selective, because the compound had little or no inhibitory activity on several endocannabinoid-metabolizing enzymes, including *N*-acylphosphatidyl-ethanolamine-selective phospholipase D, diacylglycerol lipase-α, monoacylglycerol lipase, and *N*-acylethanolamide-hydrolyzing acid amidase (Supplementary Fig. 10a–d). Moreover, ARN272 produced only a weak and incomplete inhibition of rat brain FAAH activity (Fig. 3d), and was not significantly hydrolyzed after incubation with recombinant human FAAH-1 (≈5% hydrolysis after 24h at 37°C). Consistent with these observations, administration of ARN272 (1 mg-kg^{-1}, intraperitoneal, i.p.) in mice increased plasma levels of anandamide (Fig. 3e) without changing the levels of 2-AG, OEA or PEA (Fig. 3f). The inhibitory effects of ARN272 on anandamide internalization in vitro and anandamide deactivation in vivo, along with the diminished anandamide accumulation observed in *faah-1*$^{-/-}$ mice, suggest that FLAT plays an important role in the membrane translocation of this endocannabinoid transmitter.

ARN272 attenuates nociceptive and inflammatory pain

Anandamide transport inhibitors produce a variety of CB_1-mediated responses, which include analgesia in animal models of nociceptive and inflammatory pain[24,25]. We tested therefore whether ARN272 might alleviate pain-related behaviors elicited in mice by intraplantar injection of the chemical irritant, formalin. Systemic administration of ARN272 (0.01–1 mg-kg^{-1}, i.p.) caused a dose-dependent reduction of formalin-induced pain behavior (Fig. 4a). Substantial antinociceptive activity was observed on both the first phase of formalin pain, which involves acute activation of sensory C fibers, and the second phase of formalin pain, in which sensory fiber activity is accompanied by inflammation and central sensitization (Fig. 4a). Similar effects were observed when ARN272 (0.01−3 μg per animal) was injected into the cerebral ventricles (Supplementary Fig. 11). The CB_1 antagonist AM251 suppressed the antinociceptive actions of systemic ARN272, whereas the CB_2 antagonist AM630 and the transient receptor potential vanilloid-1 (TRPV-1) antagonist AMG9810 were ineffective (all drugs administered at 1 mg-kg^{-1}, i.p) (Fig. 4b). The CB_1-mediated antinociceptive effects demonstrated by ARN272 in the formalin test are similar to those previously reported for anandamide transport inhibitors such as AM404, OMDM-1 and UCM707[24].

In another set of experiments, we evaluated the ability of ARN272 to alleviate thermal hyperalgesia and paw edema elicited in mice by the pro-inflammatory polysaccharide, carrageenan. Similarly to FAAH inhibitors[26,27], ARN272 (0.01–3 mg-kg^{-1}, i.p.) exerted anti-hyperalgesic (Fig. 4c and Supplementary Fig. 12) and anti-inflammatory effects (Fig. 4e, Supplementary Fig. 13). These were suppressed by blockade of CB_1, but not CB_2 or TRPV-1 receptors (Fig. 4d,f and Supplementary Fig. 14). ARN272 did not evoke any detectable nocifensive response when administered alone into the mouse paw (Supplementary Table 1), confirming its inability to interact productively with TRPV-1.

Finally, ARN272 did not significantly alter the binding of the cannabinoid ligands [^{3}H]-CP55940 or [^{3}H]-anandamide to rat brain membranes (Supplementary Fig. 10e). Together, the findings indicate that ARN272 elicits profound analgesic effects in mice, which result from inhibition of anandamide transport.

DISCUSSION

The functional properties of FLAT suggest that this protein is a key molecular component of the anandamide transport system in neural cells, and 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, U C M-707 and VDM-11[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 levels, and analgesia in models of nociceptive and inflammatory pain[3]. Consistent with these data and previous reports[3,20,21,23], deletion of the *faah-1* gene substantially reduced anandamide transport in mouse cortical neurons, whereas acute pharmacological blockade of FAAH activity failed to do so. While implying an important role for FLAT in anandamide transport, our findings do not rule out the possibility that additional components of the endocannabinoid transport system remain to be discovered. In this context, it is important to point out that FLAT expression did not confer [^{3}H]-2-AG or [^{3}H]-OEA transport to Hek293 cells, and administration of the FLAT inhibitor ARN272 did not increase plasma levels of 2-AG or OEA in mice, which indicates that the translocation of these lipid mediators[4,28,29] may be independent of FLAT. Because of its ability to inhibit anandamide deactivation selectively, ARN272 may be useful to differentiate the functional roles of anandamide from those of other lipid amides that are substrates for FAAH (e.g., OEA and PEA).

Multicellular organisms utilize 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 employed for this task: integral membrane transporters, such as CD36[30] and PGT[31], and lipid chaperones, such as aP2 and mal1 (fatty acid-binding protein-4 and 5, respectively)[32]. For example, membrane-bound CD36 in small-intestinal enterocytes captures dietary oleic acid and directs it toward the intracellular biosynthesis of OEA, an important gut hormone[33]. On the other hand, cytosolic aP2 in adipocytes encapsulates fatty acids derived from the circulation and partitions them toward appropriate cellular sites for storage or oxidative metabolism[32]. 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[34] (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 unique in at least two ways. First, its substrate preference and sensitivity to pharmacological agents distinguish it from other carriers for lipophilic ligands, such as serum albumin and fatty

acid-binding proteins, which are known to sequester anandamide[35–38]. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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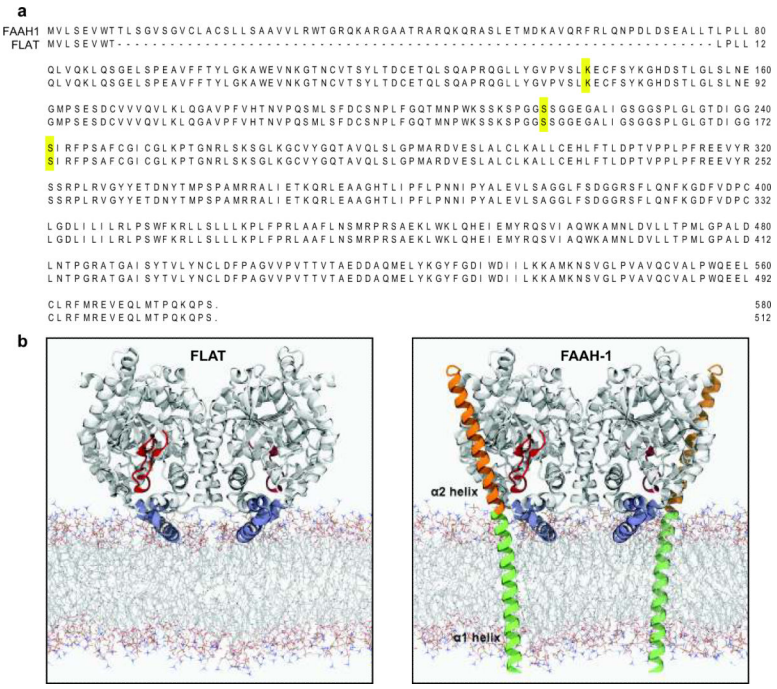

Fig. 1.

Structural properties of FLAT. (**a**) Predicted amino acid sequences of FLAT and FAAH-1; residues comprising the catalytic triad of FAAH-1 (Lys^{142}, Ser^{217} and nucleophile Ser^{241}) are highlighted. (**b**) Model of rat FLAT (*left*) based on the structure of FAAH-ΔTM (*right*), a FAAH-1 mutant lacking the α1 helix[15] which is redrawn in green for illustration purposes. Most of the α1 helix and the entire α2 helix (orange) of FAAH-1 are absent in FLAT. Both FAAH-1 and FLAT contain a membrane-binding domain (blue, FLAT residues 343–367) and an 'α2-interacting loop' (red, FLAT residues 187–210), which may interact with the α2 helix and help shield the enzyme's catalytic pocket from water. The membrane model was generated using Molecular Dynamics simulations of a 1,2-dioleoyl-*sn*-glycerol-3-phosphorylcholine bilayer[39].

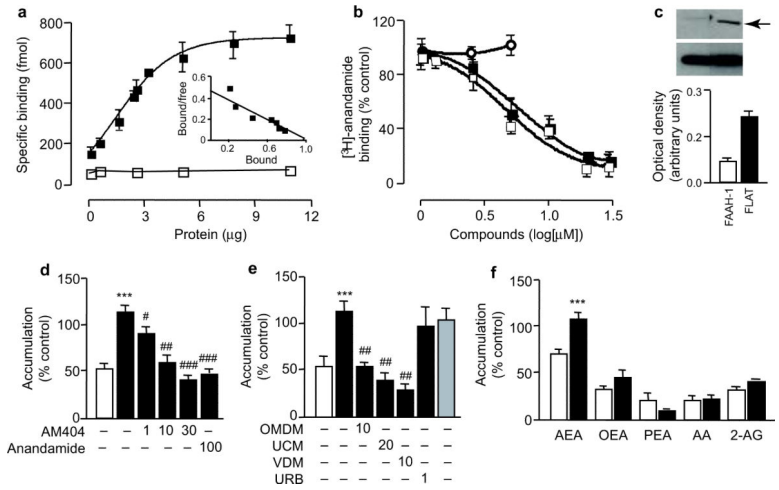

Fig. 2.

FLAT binds to anandamide and facilitates its transport into cells. (**a**) Specific binding of [^{3}H]-anandamide to rat FLAT-glutathione-*S*-transferase (GST) (closed squares) or GST alone (open squares). The inset shows a Scatchard transformation (bound - [bound/free], in nmol) of binding data. (**b**) AM404 (closed squares) and OMDM-1 (closed squares) antagonize [^{3}H]-anandamide binding to FLAT-GST, whereas URB597 (open circles) has no effect. (**c**) Cytosolic fractions of FLAT-expressing Hek293 cells contain detectable amounts of FLAT (arrow); a corresponding fraction from FAAH-1-expressing cells is shown for comparison. The lower band is β-actin. (**d**) [^{3}H]- Anandamide accumulation in control cells (vector-transfected, open bar) or FLAT-expressing Hek293 cells (closed bars) incubated with vehicle (0.01%dimethylsulfoxide), AM404 or non-radioactive anandamide (concentrations in μM). (**e**) The anandamide transport inhibitors OMDM-1, UCM-707 and VDM-11 suppress [^{3}H]-anandamide accumulation in FLAT-expressing Hek293 cells. This process is not affected by URB597 or mutation of catalytic Ser^{242} (shaded bar). (**f**) Accumulation of labeled lipids in control (open bars) or FLAT-expressing Hek293 cells (closed bars). Abbreviations: [^{3}H]-anandamide, AEA; [^{3}H]-oleoylethanolamide, OEA; [^{3}H]-palmitoylethanolamide, PEA; [^{3}H]-arachidonic acid, AA; [^{3}H]-2-arachidonoyl-*sn*-glycerol, 2-AG. Results are expressed as mean±SEM of 3-7 experiments. ***P<0.01, versus vector-transfected cells, Student's t test; $^{\#}P$<0.05; $^{\#\#}$, P<0.01; $^{\#\#\#}$, P<0.001 versus vehicle, one-way ANOVA followed by Dunnett's test.

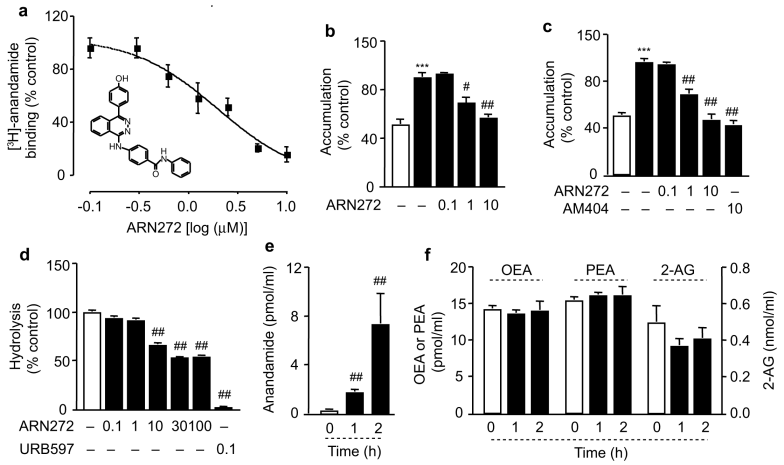

Fig. 3.

ARN272 is a competitive FLAT inhibitor. (**a**) Effects of ARN272 on [^{3}H]-anandamide binding to FLAT-GST. The inset shows the chemical structure of ARN272. (**b**) Effects of ARN272 (concentrations in μM) on [^{3}H]-anandamide accumulation in FLAT-expressing Hek-293 cells (closed bars); the open bar represents vector-transfected cells. (**c**) Effects of vehicle (open bar), ARN272 and AM404 (closed bars) on [^{3}H]-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 levels of (**e**) anandamide or (**f**) OEA, PEA and 2-AG in mice. Results are expressed as mean±SEM of 3–7 experiments. ***, P<0.001 versus vector-transfected cells, Student's t test; $^{\#}$, P<0.05 and $^{\#\#}$, P<0.01 versus vehicle; one-way ANOVA followed by Dunnett's test.

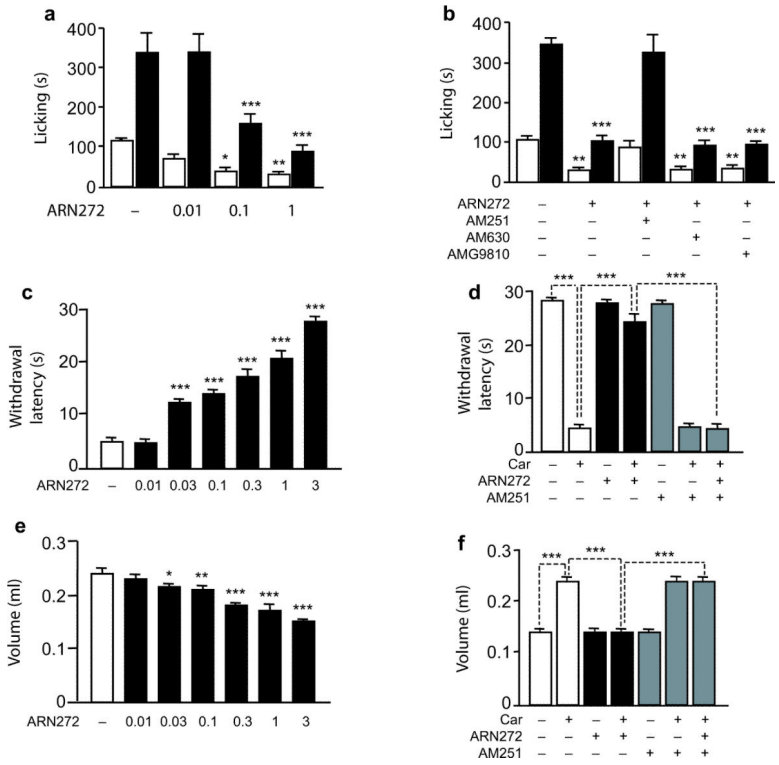

Fig. 4.

ARN272 produces CB_1-dependent antinociception in mice. Intraplantar injection of formalin (5%, 20 μL) elicited two temporally distinct phases of nocifensive behavior in mice: phase I (0–5 min, open bars) and phase II (5–45 min, closed bars). (**a**) ARN272 (doses in mg-kg^{-1}, i.p.) decreased nocifensive behavior in both phases. (**b**) The CB_1 antagonist AM251 (1 mg-kg^{-1}, i.p.) abolished the antinociceptive effects of ARN272, whereas the CB_2 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, in seconds), and (**e**) edema (volume, in ml). The CB_1 antagonist AM251 (1 mg-kg^{-1}, i.p.) suppressed the effects of ARN272 on (**d**) thermal hyperalgesia and (**f**) edema. Results are expressed as the mean±SEM of 6 mice per group. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ versus vehicle-injected controls; two-way ANOVA followed by Bonferroni's test.