Ex-vivo imaging of fatty acid amide hydrolase (FAAH) activity and its inhibition in the mouse brain

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2-AG 2-arachidonoyl glycerol
AA arachidonic acid
AM404 N-(4 hydroxyphenyl)-arachidonamide
CB1R cannabinoid receptor 1
\(\Delta^9\)-THC \(\Delta^9\)-tetrahydrocannabinol
FAAH-IR fatty acid amide hydrolase immunoreactive
FAAH KO fatty acid amide hydrolase knockout
i.v. intravenous
MGL monoacylglycerol lipase
MAFP methylarachidonyl fluorophosphonate
PC phosphatidyl choline
PI phosphatidylinositol
PLA2 phospholipase A2
TLC thin layer chromatography
WT wild-type

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Abstract:

There is recent behavioral evidence that FAAH inhibitors produce a sub-set of cannabinoid receptor agonist effects, suggesting both anandamide-specific behavioral functions, and possible regional differences in FAAH inhibitory effects. Here we introduce a novel imaging method to quantify regional differences in brain FAAH activity. Upon intravenous \(^{3}\text{H}\)anandamide administration, brain FAAH activity generates \(^{3}\text{H}\)arachidonic acid, which is promptly trapped in membrane phospholipids. As a result, WT brains accumulate tritium in a regionally specific manner that is dependent upon regional FAAH activity, while brains from FAAH KO mice show a uniform \(^{3}\text{H}\)anandamide distribution. Increasing doses of anandamide+\(^{3}\text{H}\)anandamide fail to alter regional tritium accumulation, suggesting insensitivity towards this process by anandamide-induced changes in regional cerebral blood flow. Regional tritiated metabolite levels in WT brains were highest in the somatosensory and visual cortex, and thalamus. Treatment with methylarachidonyl fluorophosphonate (MAFP) (1 mg/kg, i.p.), reduced regional tritium accumulation in the somatosensory and visual cortex (p<0.01), and at higher doses, the thalamus (p<0.05). The selective FAAH inhibitor CAY10435, while having similar efficacy as MAFP in reducing tritium in the thalamus, somatosensory and visual cortex, also reduces caudate putamen and cerebellum (p<0.01) activity. These data indicate FAAH activity generates heterogeneous regional accumulation of \(^{3}\text{H}\)anandamide and metabolites, and suggest the modulation of endocannabinoid tone by FAAH inhibitors depends upon not only the dose and compound used, but also the degree of FAAH expression in the brain regions examined. This imaging method determines regionally-specific FAAH inhibition and can elucidate the \textit{in vivo} effects of pharmacological agents targeting anandamide inactivation.
Δ⁹-tetrahydrocannabinol (Δ⁹-THC), a cannabinoid receptor 1 (CB1R) agonist ((Matsuda et al., 1990), promotes well documented behavioral and physiological effects in animals and humans (For review, see (Pertwee, 2005)). Similar to Δ⁹-THC, exogenously administered anandamide (Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), two endogenous CB1R ligands, reduce rectal temperature and spontaneous activity, and promote analgesia in mice and rats. However, due to the prompt inactivation of anandamide and 2-AG in vivo, these cannabimimetic effects are transient (Crawley et al., 1993; Frade and Mechoulam, 1993; Mechoulam et al., 1995; Stein et al., 1996).

Anandamide and 2-AG are degraded in vivo through independent enzymatic pathways. FAAH knockout (FAAH KO) mice have 15 fold higher endogenous anandamide levels than wild-type (WT) mice, while maintaining similar endogenous 2-AG levels (Cravatt et al., 2001; Lichtman et al., 2002). FAAH, therefore, plays a central role in anandamide degradation in the mouse, while 2-AG is degraded primarily by other enzymes, such as monoacylglycerol lipase (MGL)(Dinh et al., 2002; Lichtman et al., 2002). Unfortunately, the study of MGL-mediated degradation of 2-AG in vivo is hindered by the current lack of selective MGL inhibitors and transgenic MGL knockout mice.

The transient behavioral effects of exogenous anandamide are enhanced through the genetic deletion or pharmacological inhibition of FAAH. Upon [³H]anandamide administration to WT mice, [³H]anandamide is rapidly distributed systemically, with less accumulation in brain than most peripheral tissues. [³H]anandamide is metabolized within one minute upon entry into the brain (Willoughby et al., 1997). FAAH KO mice, however, cannot efficiently metabolize
anandamide. As a result, they possess elevated levels of endogenous anandamide, while exogenous anandamide evokes prolonged CB1R-mediated effects (Cravatt et al., 2001). Selective FAAH inhibitors URB597 (Kathuria et al., 2003) and OL-135 (Lichtman et al., 2004), also increase brain endogenous anandamide levels, and produce CB1R-mediated behaviors in rodents. URB 597 produces anxiolytic effects and moderate analgesia, without inducing catalepsy, hypothermia, or hypophagia (Kathuria et al., 2003). OL-135 additionally inhibits nociception and reduces rectal temperature, again without deleterious motor effects (Lichtman et al., 2004). The regional selectivity of FAAH inhibitors might be exploited to produce drugs with potent anxiolytic and analgesic effects but without memory and motor impairments, produced by direct CB1R agonists (For review, see (Cravatt and Lichtman, 2004)). A likely mechanism for behavioral selectivity of URB597 and OL-135 is that these drugs do not inhibit FAAH to the same degree in brain regions supporting the various behaviors.

While the processes of anandamide accumulation (For review, see (Glaser et al., 2005)) and inactivation (For review, see (Cravatt and Lichtman, 2003)) have been extensively examined in cultured cells and tissue homogenates, there are few studies of these processes in intact tissue. Accumulation of radioactivity in rat brain slices following exposure to [14C]anandamide is heterogeneous, and is blocked by the FAAH and putative transport inhibitor AM404 (Giuffrida et al., 2001). A recent study in goldfish retina documented a pattern of tritium accumulation following an incubation in [3H]anandamide overlaps that of FAAH immunoreactivity (FAAH-IR). Dependence of tritium accumulation upon FAAH activity was confirmed by its blockade by FAAH inhibitors (Glaser et al., 2005).
Following hydrolysis of $[^3]H$anandamide by FAAH in vitro, liberated $[^3]H$AA is predominantly incorporated into phospholipids (Deutsch and Chin, 1993; Willoughby et al. 1997, Glaser et al., 2005). In the present study, we evaluated the potential of this “metabolic trapping” for in vivo autoradiographic studies of FAAH activity. We hypothesized that brain regions with high FAAH activity would have the most regional retention of radioactivity, which would comprise radioactive phospholipids. A concentration gradient generated by FAAH activity would drive robust $[^3]H$anandamide accumulation, with the blood supply, and possibly neighboring regions with limited metabolic activity, as the $[^3]H$anandamide source. In contrast, we expected FAAH KO brains, which do not metabolize anandamide (Cravatt et al., 2001), to homogenously accumulate $[^3]H$anandamide, as no concentration gradient would be maintained. Reversible hydrophobic interactions with membranes and proteins could dominate, with radioactivity continually redistributed within FAAH KO brains. This process would allow observation of rates of FAAH activity in brain structures and examination of the effects of FAAH inhibitors. This information, in conjunction with behavioral testing, could facilitate development of clinically useful FAAH inhibitors.

Methods

Animals

C57BL/6 mice (Taconic Farms, Germantown, NY) and FAAH KO mice (a generous gift from Dr. B. Cravatt) weighing 20 to 30g were used in all experiments. Mice were given free access to food and water, kept in LD 12:12, and were maintained according to the federal guidelines for the care and use of animals. These studies were approved by the institutional review committee.

Compounds
Anandamide, arachidonic acid (AA), methyl arachidonyl fluorophosphonate (MAFP), and 1-oxazolo[4,5-b]pyridin-2-yl-1-dodecanone (CAY10435) were purchased from Cayman Chemical (Ann Arbor, MI). Radiolabeled anandamide [arachidonyl 5,6,8,9,11,12,14,15-³H] and arachidonic acid [arachidonyl 5,6,8,9,11,12,14,15-³H] were purchased from American Radiolabeled Chemicals (St. Louis, MO).

**Time Course Assays**

To establish the optimal time point for imaging, WT mice were injected in the tail vein with 0.5 mCi /kg [³H]anandamide plus 10mg/kg anandamide, dissolved in a 1:1:18 mixture of ethanol: emulphor: saline, and sacrificed 30s to 60 minutes later. The brains were isolated and blood samples collected. All tissues were immediately chilled on ice, weighed, homogenized in 0.1M phosphate buffer (PB, pH 7.3), and extracted with two volumes of 1:1 chloroform: methanol. Samples were centrifuged, and the chloroform layer collected. A portion of the chloroform layer was assayed for total tritium levels (representing brain incorporation of [³H]anandamide and its metabolites), and the remainder was spotted on a Baker-flex silica gel IB-F TLC plate (J.T. Baker Inc., Phillipsburg, NJ) for thin layer chromatography (TLC). The solvent system for the TLC was 6: 3: 1 ethyl acetate: hexane: acetic acid. The resulting TLC was cut into 1 cm squares, 200 µL of chloroform was added, followed by 12 mL UltimaGold XR scintillation fluid (Packard Bioscience). Samples were quantified using a Tri-Carb 1600 TR betascintillation counter (Packard Bioscience). Data from three to six mice were averaged for each time point, and statistical analysis, including standard error and 2-tailed t-tests performed with Microsoft Excel. Graphs with standard error were generated using SigmaPlot 2000 (SPSS, Chicago, IL).

**Ex vivo Autoradiography**
10 mg/kg anandamide and 0.75 mCi/kg $[^3]$H]anandamide were dissolved in a 1:1:18 mixture of ethanol: emulphor: saline, and intravenously administered (via tail vein) to both WT and FAAH KO. In dose response studies, 1, 5, or 10 mg/kg anandamide + 0.75 mCi/kg $[^3]$H]anandamide was administered to WT mice. 10 mg/kg AA + 0.75 mCi/kg $[^3]$H]AA was administered to WT mice as a control for peripheral metabolite accumulation in the brain. For inhibitor studies, either CAY10435 or MAFP was co-administered i.v. at the dose indicated with 10 mg/kg anandamide + 0.75 mCi/kg $[^3]$H]anandamide. Thirty minutes following the injection, the mice were sacrificed and their brains promptly transferred to a beaker containing iced saline, in order to minimize further diffusion and protein-mediated processes upon anandamide and its metabolites. Half of the cerebellum from each mouse was processed for scintillation counting and TLC as described above. The remaining brain samples were transferred into 2% paraformaldehyde + 2% glutaraldehyde in PB on ice for one hour. The brains were then washed three times with iced PB, and cryoprotected overnight at 4°C in 30% sucrose in PB. 40 µm serial cryosections were made and promptly fixed for five minutes in 2% OsO4 in PB. The slides were rinsed in distilled water, and desiccated for at least 3 days. Images of surface tritium levels were acquired under high resolution and full-field for eight to 18 hours using the BetaImager (Biospace, France). A standard slide was included to control for variability between scans. Three to six mice were used for each condition. Light micrographs of sections were taken with a Zeiss epifluorescence microscope and a Kodak MDS-290 digital camera (Eastman Kodak, New Haven, CT).

**Autoradiography Analysis**

Surface radioactivity (cpm/mm²) in regions of interest was quantified using Beta Vision+ image analysis software (Biospace, France). Average regional accumulation was determined using
Excel (Microsoft Corp., Seattle, WA), Regional surface activities were normalized by dividing each regions activity by piriform cortex of the same mouse. The latter region was selected as a reference region on the basis of having the lowest overall counts. Normalizing data in this way corrects for differences in overall brain tritium uptake between mice. Significance between WT and FAAH KO among inhibitor doses was determined using a one-way ANOVA with Dunnett's post test in GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California). Graphs showing the normalized regional averages and standard error were generated with SigmaPlot 2000 (SPSS, Chicago, IL).

**FAAH Activity Assay**

The enzyme assay was conducted as described earlier (Deutsch and Chin, 1993). MAFP (Cayman Chemical) was administered in the tail vein of six WT mice at each dose indicated in a vehicle of 1:1:18 ethanol: emulphor: saline. Mice were sacrificed 30 min later, and their brains immediately frozen on dry ice. Dissections were performed on the frozen tissue, and the hippocampus, thalamus, and cerebellum were isolated. Tissue was homogenized in Tris + 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6. Protein concentrations of all homogenates were determined by BCA assay (Pierce Biotechnology, Inc., Rockford, IL).

Incubations were performed in triplicate at 37°C while shaking. Each sample contained 10 ml of 50 mg/ml fatty acid-free BSA (Sigma–Aldrich), 330 µg of regional homogenate, and 30 µM anandamide (Cayman Chemical) 1 0.01 mCi of 120 mCi /mM arachidonoyl ethanolamide [ethanolamine-1,2-14C] (NEN). Negative control tubes contained FAAH KO brain homogenate. The reactions were terminated after 30 min by the addition of 2 volumes chloroform:methanol (1:1). Samples were spun, and the aqueous phase was measured by liquid scintillation counting.
Data were graphed by using Sigma Plot as percent inhibition of control homogenate activity. Experiments were repeated three times. Representative curves are shown.

**Trapping Mechanism for Imaging In Vivo FAAH Activity**

In order to visualize *in vivo* FAAH activity, we injected mice with [$^3$H-arachidonoyl] anandamide. In cells containing FAAH, labeled anandamide is converted into ethanolamine and [$^3$H]AA, which is rapidly incorporated into the phospholipid “sink”. The regional deposition of tritium thus reflects the regional activity of FAAH. In the brain, AA is efficiently incorporated into membrane phospholipids (Deutsch and Chin, 1993). This process is mediated by acyl CoA synthetase and acyl transferase and is the predominant metabolic pathway for AA in the rodent brain (Sun, 1977). AA is incorporated primarily into phosphatidyl inositol (PI), and phosphatidyl choline (PC) (Nariai et al., 1993) (See Fig. 1). The liberation of AA from membrane phospholipids, mediated by phospholipase A$_2$ (PLA$_2$), is much slower than its incorporation; the half-lives of arachidonyl PI and PC in the rat brain are 3.4 and 2.9 hours, respectively (Rapoport, 1999). Therefore, using a time point that allows for near-complete incorporation of radiolabeled AA into membranes without a significant effect of PLA$_2$ (For review, see (Robinson et al., 1992)), it was possible to image incorporation of exogenous AA into brain lipids (Giovacchini et al., 2002).

**Results**

**Determination of imaging conditions**

In order to determine the feasibility of imaging FAAH activity in the mouse brain, it is important that sufficient tritium enters the brain following [$^3$H]anandamide administration. Tritium
accumulation in the brain is affected by multiple processes, including the dose of \[^3\text{H}\]anandamide given, its route of administration, and the ability of \[^3\text{H}\]anandamide to cross the blood brain barrier, accumulate in peripheral tissues, and be metabolized in the brain and peripheral sites. Since intravenously administered anandamide accumulates in the brain, and is efficiently metabolized in both the brain and peripheral tissues of WT mice (Willoughby et al., 1997), the optimal time for imaging will be dictated by the time at which maximal tritium accumulation is observed in the brain, with the bulk of \[^3\text{H}\]anandamide already metabolized.

A time course study was performed, where the degree of tritium accumulation and metabolism in WT brain and blood samples was used to determine the optimal time frame for autoradiograph studies. The total tritium, representing the accumulation of \[^3\text{H}\]anandamide and metabolites, increased (p<0.01) between 1 and 5 min until 2.38 +/- 0.22% of the injected dose accumulated in the brain (Fig. 2a), with no further changes at later time points (Fig. 2a and 2b) (all discussed increases and decreases in compound levels are significant (p<0.05), unless otherwise stated). The disappearance of tritium from the blood was extremely fast, with reductions (p<0.01) occurring between 1 and 5 minutes (Fig. 2b).

The degree of \[^3\text{H}\]anandamide metabolism in blood and brain samples was determined by thin layer chromatography. Reductions in \[^3\text{H}\]anandamide levels occurred in the blood within 1 to 15 minutes, while increases in levels of its metabolite, \[^3\text{H}\]AA were observed between 30s and 15 minutes (Fig. 3a). In the brain, however, no changes in the proportion of \[^3\text{H}\]anandamide and metabolites occurred during the time course study. At all times examined, the majority of brain
tritium was in metabolite form (Fig. 3b). Since the bulk of peripheral metabolism was complete within 15 minutes, we chose a time point of 30 minutes for the remaining studies.

In order to ensure that anandamide dose had minimal effect upon the degree of tritium accumulation in the brain, WT mice were administered increasing doses of unlabeled anandamide, and their brain tissue analyzed for tritium accumulation 30 min later. The amount of anandamide and [3H]anandamide and metabolite incorporation was proportional to the administered dose (Fig. 2c). When 10 mg/kg anandamide plus [3H]anandamide was administered to FAAH KO mice, less tritium was observed in the blood than in WT, while brain tritium levels between the two mouse types did not vary. Since anandamide dose did not alter tritium accumulation, 10 mg/kg anandamide was administered in all imaging experiments, unless otherwise stated.

**Imaging WT and FAAH KO brains**

Since the optimal imaging conditions were determined, the regional accumulation of [3H]anandamide and its metabolites was examined in WT and FAAH KO mice. Following the i.v. administration of 10 mg/kg anandamide and 0.75 mCi/kg [3H]anandamide, brains were isolated 30 min later, and serial sections analyzed by autoradiography. Following tritium administration, WT brain tritium incorporation was regionally heterogeneous, while that in FAAH KO brains appeared homogeneous (Fig. 4). In order to control for the peripheral metabolism of [3H]anandamide and subsequent brain accumulation of [3H]AA, a control group of WT mice were administered 10 mg/kg AA plus [3H]AA. Following [3H]AA administration,
brain tritium accumulation was less pronounced than when \([^{3}H]\)anandamide was administered (Fig. 4), suggesting a poorer brain penetration of \([^{3}H]\)AA than \([^{3}H]\)anandamide.

Regional analysis of FAAH KO brain autoradiographs demonstrated no variability in regional tritium distribution. In contrast, a heterogeneous pattern was seen in WT brains, with more radioactivity in the somatosensory cortex, thalamus, and visual cortex of WT mice. The normalized activity in the caudate putamen, hippocampus, hypothalamus, periaqueductal gray, and substantia nigra of WT did not vary from FAAH KO regions (Fig. 5b).

In order to address anandamide-induced changes in regional cerebral blood flow, and its potential contribution to both regional tritium delivery, and regional tritium accumulation in WT mice, mice were administered increasing doses (1-10 mg/kg) of anandamide plus \([^{3}H]\)anandamide, and their brain autoradiographs analyzed as described above. Increasing doses of anandamide did not affect regional tritium accumulation in WT brains (Fig. 5a).

**Imaging regional effects of FAAH inhibitors**

Since the degree of FAAH expression varies among brain regions, imaging the regional inhibition in FAAH activity could provide valuable information of the *in vivo* effects of such compounds. In order to examine inhibitor-induced changes in anandamide metabolism, WT mice were co-administered anandamide plus \([^{3}H]\)anandamide and increasing doses of MAFP or CAY10435. After 30 min, most of the brain was isolated and processed for imaging. Half of the cerebellum and a blood sample were analyzed by traditional biochemical methods, scintillation counting, and thin layer chromatography. These two methods were used to confirm the sensitivity of this novel imaging method in determining regional FAAH activity. When WT mice
were co-administered 10 mg/kg anandamide plus $[^3]H$anandamide and 1mg/kg MAFP or CAY10435, there was less tritium in blood samples. While higher doses of MAFP failed to produce any further reductions, 8 mg/kg CAY10435 further reduced blood tritium levels. Neither compound affected tritium incorporation in the cerebellum at any dose tested (Fig. 6a and 6b).

Thin layer chromatography analysis indicated increasing doses of MAFP decreased the proportion of $[^3]H$AA in the blood, while 8 mg/kg MAFP increased the level of nonmetabolized $[^3]H$anandamide (Fig. 7a). Doses of 4 mg/kg to 12 mg/kg CAY10435 reduced $[^3]H$AA levels in the blood. No dose of CAY10435 examined increased $[^3]H$anandamide blood levels (Fig. 7b). When administered to FAAH KO mice, 8 mg/kg MAFP reduced blood $[^3]H$AA levels. CAY10435 had no effect upon blood tritium levels in FAAH KO (Fig. 7c).

While 4 mg/kg MAFP reduced $[^3]H$AA levels in the cerebellum, increases in nonmetabolized $[^3]H$anandamide, and reductions in $[^3]H$phospholipid levels were only observed following the administration of 8 mg/kg MAFP (Fig. 7d). CAY10435 failed to produce changes in $[^3]H$anandamide levels at any dose tested, but reduced some $[^3]H$metabolite levels at 8mg/kg and 12 mg/kg doses (Fig. 7e). Neither inhibitor produced any changes in tritium levels in the FAAH KO cerebellum (Fig. 7f).

Analysis of autoradiographs indicated MAFP and CAY10435 affected the regional tritium accumulation pattern in WT brains. MAFP reduced normalized activity in the caudate putamen, cerebellum and hypothalamus at some doses. At every dose, reductions (p<0.01) in normalized activity were observed in the somatosensory cortex and visual cortex. The thalamus, however, was affected only at doses above 1 mg/kg (Fig. 8a).
Similar to MAFP, while CAY10435 produced reductions (p<0.01) in normalized activity in the somatosensory cortex and visual cortex at every dose, effects upon the thalamus were only observed at doses of 4mg/kg and higher. Administration of CAY10435 also produced highly reductions (p<0.01) in normalized activity in the caudate putamen and cerebellum, and reductions in the hypothalamus and periaqueductal gray (Fig. 8b), the latter of which was not affected by MAFP (Fig. 8a). 12 mg/kg CAY10435 and 8 mg/kg MAFP did not vary the normalized regional activity in FAAH KO brains (data not shown).

To confirm the regional changes in tritium accumulation are indeed caused by a regional inhibition of FAAH, WT mice were intravenously administered increasing doses of the irreversible inhibitor MAFP, and their brains isolated 30 min later. Regional homogenates were generated, and the rates of \(^{[14}C\)anandamide metabolism compared to homogenates generated from untreated mice. Similar to the imaging data shown in Fig. 8a, reductions in thalamic homogenate FAAH activity were observed following administration of 8 mg/ kg MAFP, but not 1 mg/kg MAFP (Fig. 9). In the cerebellum, both the homogenate and imaging assays detected reductions in anandamide metabolism following the administration of 1 mg/kg MAFP. The modest hippocampal tritium accumulation observed in the imaging assay likely obscured any effects of MAFP in that region. The homogenate assay, however, detected reductions in hippocampal anandamide hydrolysis following inhibitor administration.

Discussion

The rapid incorporation of AA into slowly metabolized phospholipid pools has been exploited for biochemical analysis (For review, see (Robinson et al., 1992).) and for imaging (For review, see (Rapoport, 2003)) accumulation of AA by the brain. Here, we show brain FAAH activity
can be imaged after the administration of [3H]anandamide, since only cells that possess FAAH can accumulate phospholipids labeled with [3H]AA. In FAAH KO mice, our data suggest [3H]anandamide can diffuse freely without being metabolized and trapped.

*Initial [3H]anandamide accumulation and stability of brain [3H]metabolites*

Following intravenous administration, blood [3H]anandamide levels decreased rapidly (Fig. 2 and 3), and over 2% of the injected dose accumulated in the brain as labeled metabolites. Similar prompt metabolism of administered anandamide was reported by Willoughby et al. (1997). After 30 min, blood from FAAH KO mice contained less tritium than WT mice, suggesting reduced peripheral accumulation of [3H]anandamide in FAAH KO mice. WT and FAAH KO brains had similar total tritium levels, but exhibited pronounced differences in the accumulation patterns.

WT brain [3H]phospholipid levels did not vary from 5 to 60 min (Fig. 3), suggesting trapped membrane tritium is not appreciably metabolized within this time frame. This is consistent with the stability of membrane incorporated [14C]AA one hour following intravenous administration (For review, see (Rapoport, 2003)), and a half-life of [14C]phospholipids of approximately three hours (Rapoport, 1999) in rat brains. We image brains within 30 min of anandamide administration, which is similar to imaging studies examining [14C]AA brain incorporation (DeGeorge et al., 1989). Our results indicate that the observed tritium incorporation is driven by FAAH activity and is minimally affected by downstream metabolism of [3H]metabolites incorporated into phospholipids, similar to the situation in cultured cells (Day et al., 2001; Deutsch et al., 2001).
Anandamide dose and regional cerebral blood flow (rCBF)

Anandamide affects both peripheral and cerebral circulatory systems (For review, see (Kunos et al., 2000)). Notably, 3-30 mg/kg anandamide i.v. produced dose-dependent reductions in rat rCBF in the amygdala, hippocampus, neocortex, and caudate, without affecting the dentate gyrus, thalamus, hypothalamus, and substantia nigra (Stein et al., 1998). Since anandamide co-administered with [3H]anandamide may affect rCBF and regional [3H]anandamide delivery, a dose response study was performed. Upon administration of 1 to 10 mg/kg anandamide+[3H]anandamide, there was a proportional increase in WT blood and cerebellar tritium levels (Fig. 2c), with no change in normalized regional activity (Fig. 5a), indicating anandamide-mediation of rCBF minimally affects regional [3H]anandamide. Arachidonate accumulation is also independent of rCBF and is instead driven by metabolism (DeGeorge et al., 1989) (For review, see (Robinson et al., 1992)).

WT and FAAH KO brain image analysis

Following [3H]anandamide administration, little tritium was trapped in phospholipids of FAAH KO mice (Fig. 7) consistent with a greatly reduced rate of [3H]AA generation from [3H]anandamide in these animals (Cravatt et al., 2001). Instead, the homogeneous distribution of radioactivity presumably reflects mostly nonspecific binding of [3H]anandamide to membranes (Fig. 5). It is unlikely that in vivo binding of [3H]anandamide at cannabinoid receptors or transport can be imaged, since the low-affinity binding at these sites is likely obscured by nonspecific membrane binding. Therefore, it is expected that processes significantly altering regional FAAH activity predominantly influence these imaging data.
The WT $[^3\text{H}]$metabolite pattern (Fig. 4 and 5) was heterogeneous, and appeared similar to patterns in rat brain slices exposed to $[^{14}\text{C}]$anandamide in vitro (Giuffrida et al., 2001). WT tritium accumulation was higher (p<0.01) than FAAH KO in the somatosensory cortex, thalamus, and visual cortex. WT and FAAH KO regions with similar accumulation included the caudate putamen, hippocampus, hypothalamus, periaqueductal gray, and substantia nigra. The lower level of accumulation in these areas does not imply an absence of FAAH activity, since tritium in the brain is predominantly $[^3\text{H}]$phospholipids (Fig. 3b) which must arise from hydrolysis of $[^3\text{H}]$anandamide to $[^3\text{H}]$AA.

Brain images of WT mice given $[^3\text{H}]$AA rather than $[^3\text{H}]$anandamide showed less accumulation (Fig. 4) and different regional deposition (data not shown). The lower accumulation is consistent with the notion that the negatively charged AA has more difficulty crossing the blood-brain barrier than the neutral anandamide. The finding that $[^{14}\text{C}]$AA accumulation is greatest in regions lacking a blood-brain barrier (DeGeorge et al., 1989) also supports this idea. The different regional deposition reflects the necessary involvement of FAAH in generating radioactivity patterns for $[^3\text{H}]$anandamide, but not $[^3\text{H}]$AA.

Intriguingly, WT $[^3\text{H}]$metabolite distribution did not precisely correspond to the FAAH-IR pattern of this mouse strain (Egertova et al., 2003). While FAAH-IR and regional activity were both robust in the thalamus and somatosensory cortex, FAAH-IR was more pronounced than activity in the hippocampal CA1 and CA3 regions, but less pronounced in the visual cortex. Our
autoradiographs measure net flux of anandamide through FAAH, whereas FAAH-IR studies indicate the presence of all protein reactive with FAAH antibodies, some of which may be covalently-modified, or sequestered from its substrate. In addition, regional net flux of FAAH may be affected by binding protein(s) and/or transporter(s), as suggested by biochemical studies. Contributions of these factors to the regional availability of FAAH substrates may remain elusive until the relative proteins have been cloned.

Imaging regional FAAH inhibition

FAAH inhibitors might be therapeutically complementary to cannabinoid receptor agonists, because unlike the latter they would increase cannabimimetic tone only where anandamide production is physiologically activated. FAAH inhibitors may preferentially elicit some cannabinoid-like behaviors (Lichtman et al., 2004; Kathuria et al., 2003). While this sub-set of cannabimimetic effects may be produced by differences in regional inhibitor sensitivity, it may also be influenced by regional rates of anandamide synthesis. We imaged [³H]anandamide metabolism in the presence of the FAAH inhibitors MAFP and CAY10435. MAFP is a classical irreversible inhibitor of FAAH (De Petrocellis et al., 1997; Deutsch et al., 1997), and PLA₂ (Street et al., 1993). CAY10435 is a selective reversible FAAH inhibitor with minimal effect upon other known serine hydrolases (Boger et al., 2000).

MAFP and CAY10435 reduced the tritium concentration in WT blood at 30 min (Fig. 6a) and both compounds reduced tritiated metabolite levels in cerebellum as well as blood, showing that FAAH was inhibited in cerebellum (Fig. 7). The higher potency of MAFP in the periphery than brain is evidenced by ED₅₀ estimates of 4 mg/kg i.p. in mouse liver and >10 mg/kg in brain
(Quistad et al., 2001). CAY10435 reduced cerebellar tritiated metabolite levels only at high
doses (Fig. 7e), and appears to be a weaker inhibitor in vivo. It is important to note that only
nearly total FAAH inhibition, able to prevent complete anandamide metabolism within 30 min,
would increase [³H]anandamide levels, and therefore TLC and scintillation counting of tissue
lack sensitivity to partial FAAH inhibition. CAY10435 was without affect in blood or
cerebellum of FAAH KO mice (Fig. 7c), confirming with the postulated selectivity of this
compound for FAAH. MAFP, did not affect cerebellum in FAAH KO mice (Fig. 7f), but did
significantly reduce [³H]AA blood levels (Fig. 7c), possibly via peripheral metabolic pathways
including PLA₂ or cyclooxygenase-2 (Weber et al., 2004).

Inhibition of FAAH activity in WT mice by MAFP and CAY10435 is regionally selective.
MAFP reduced (p<0.01) normalized activity in the somatosensory and visual cortex at every
dose tested, while the thalamus was inhibited only at higher MAFP doses (Fig. 8a). FAAH
activity assays confirmed the dose-dependent regional effects of MAFP (Fig. 9), and outlined the
shortcomings of each method in detecting FAAH inhibition. Activity assays are sensitive, but
require large amounts of tissue and are limited to brain structures that can accurately be isolated.
Autoradiography, while not limited by structure size, has reduced sensitivity in regions with
modest tritium accumulation, such as the hippocampus.

Like MAFP, CAY10435 (p<0.01) reduced activity in the somatosensory and visual cortex at all
doses, and thalamic activity at the highest dose (Fig. 8b). However, CAY10435 also consistently
inhibited FAAH in the caudate putamen and cerebellum, and at some doses, the hypothalamus
and periaqueductal gray. Since the degree of inhibitor efficacy is directly related to the amount
of enzyme present, the unique regional patterns produced by MAFP and CAY10435 could be a result of dose-related changes in compound bioavailability in addition to regional differences in FAAH expression. These imaging data suggest that FAAH inhibitors, when given at sub-maximal doses, are least effective in brain regions where FAAH expression is most abundant, such as the visual and somatosensory cortex (Egertova et al., 2003). Concurrent inhibition of other pharmacological targets could also contribute towards regional heterogeneity.

This is the first autoradiography study of FAAH activity and its inhibition in vivo. We show that regional [³H]anandamide metabolite accumulation in WT brains is heterogeneous, and dependent upon FAAH activity. The basis of the biochemical image is quasi-irreversible incorporation into phospholipids of AA generated by the action of FAAH on anandamide. Our data are consistent with the notion that FAAH activity is the rate-limiting determining step in this process, and that regional tritium deposition reflects the regional rate of anandamide hydrolysis. However, it is possible that factors such as local permeability barriers to anandamide diffusion to intracellular FAAH contribute to the images.

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References


Footnotes

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Figure Legends

Fig. 1  Trapping mechanism for \([^3\text{H}]\)anandamide metabolites. The pathway of anandamide degradation and metabolite recycling is represented, with thick arrows indicating prompt reactions, and thin arrows indicating gradual reactions. When \([^3\text{H}]\)anandamide is administered, this pathway provides a tritium trapping mechanism for imaging FAAH activity. By imaging at a time following the majority of tritium incorporation into membranes, but prior to appreciable tritium release from membranes by PLA\(_2\), it is possible to temporally isolate \([^3\text{H}]\)anandamide metabolite incorporation from downstream events in the brain. * = tritium. (For review of both major and alternate arachidonic acid metabolic pathways, see (Rapoport, 2003)).

Fig. 2  Time course and dose response studies of \([^3\text{H}]\)anandamide and metabolite accumulation in the blood and brain. (a) Percent injected dose / gram accumulation in WT brain following the i.v. administration of \([^3\text{H}]\)anandamide. The percent \([^3\text{H}]\)anandamide accumulating in the brain increased \((p<0.05)\) between one and five minutes, and did not vary at longer time points \((p>0.5)\). (b) Time course study of tritium accumulation in blood and brain following intravenous administration of 10 mg/kg anandamide + \([^3\text{H}]\)anandamide. The level of \([^3\text{H}]\)anandamide and metabolites in the blood decreased during the first five minutes \((p<0.01)\). At 30 minutes, a slight increase was observed \((p<0.05)\). Tritium levels in the brain (inset) increased \((p<0.05)\) between one and five minutes, and did not vary at subsequent time points \((p>0.05)\). (c) \([^3\text{H}]\)anandamide dose response study. WT mice were intravenously administered increasing doses (1 - 10 mg/kg) unlabeled anandamide and \([^3\text{H}]\)anandamide. After 30 min, brain tritium levels were measured, and subsequently, total anandamide accumulation was determined. The accumulation of anandamide in the brain increased proportionally to the administered dose in WT mice, with
FAAH KO mice given 10 mg/kg [$^3$H]anandamide having lower blood tritium levels (p<0.05) than their WT counterparts.

Fig. 3 *Time course of [$^3$H]anandamide and metabolite levels in the blood (a) and brain (b).* (a). The level of [$^3$H]anandamide (●) in the blood was reduced between one and 15 minutes (p<0.05), while the level of [$^3$H]AA (○) increased (p<0.05) between 30 seconds and one minute, and five and 15 minutes. In addition, there was a small increase in [$^3$H] phospholipids (▼) in the blood in the first 5 minutes (p<0.01), and later between 30 and 60 minutes (p<0.05) following injection. (b). The majority of brain tritium was in the form of tritiated metabolites incorporated into phospholipids (▼). While no changes in the levels of [$^3$H]anandamide (●) or [$^3$H]AA (○) (p>0.05) was observed at any of the time points studied, an increase (p<0.05) in [$^3$H] phospholipids occurred between one and five minutes.

Fig. 4 *Distribution of radioactivity following an injection of [$^3$H]anandamide in mice.* [$^3$H]anandamide and its metabolites accumulated in a heterogeneous pattern in WT mice (Row 2), with the most accumulation observed in the cortex and thalamus regions. In sharp contrast, FAAH KO (Row 3) mouse brains had a more homogenous distribution of tritium. *Distribution of radioactivity following an injection of [$^3$H]AA in WT mice (Row 4).* The pattern of [$^3$H]AA accumulation was faint relative to patterns produced following the administration of [$^3$H]anandamide (Rows 2 and 3). Light micrographs of tissue sections used for regional quantization are presented in Row 1. Regions: caud p- caudate putamen; cere- cerebellum; hippo- hippocampus; hyp- hypothalamus; ol- anterior olfactory nucleus; pag- periaqueductal
gray; pir- piriform cortex; sn- substantia nigra; somat- somatosensory cortex; thal- thalamus; vis- visual cortex.

Fig. 5 *Comparison of regional accumulation of \[^3\text{H}\]anandamide and its metabolites in mouse brains* (a) in a WT \[^3\text{H}\]anandamide dose response study, and b) WT and FAAH KO brains. a) WT mice administered increasing doses (1-10 mg/kg and approximately 20 \(\mu\text{Ci}\)) \[^3\text{H}\]anandamide, all exhibited similar regional normalized activity, with no dose dependent effects (p>0.05) in any region. b) WT mice had a heterogeneous accumulation pattern with more tritium (p<0.01) than FAAH KO brains in the somatosensory cortex, thalamus, and visual cortex. All other WT brain regions were not different from those in FAAH KO mice (p>0.05). The regional quantization of KO mice confirmed that the distribution of \[^3\text{H}\]anandamide was homogeneous in all regions examined, with no differences (p>0.05) in regional activity.

Fig. 6 *Tritium levels in blood and cerebellum of WT mice following the i.v. co-administration of 10 mg/kg anandamide +[^3H]anandamide and FAAH inhibitors*. (a) The percent tritium in the blood (●) decreased (p<0.01) upon the co-administration of 1 mg/kg MAFP, with no further changes (p>0.05) observed in blood tritium levels at higher doses. No changes were observed in brain (○) tritium levels (p>0.05) following the co-administration of 1-8 mg/kg MAFP. (b) Levels of \[^3\text{H}\]anandamide and metabolites in blood (●) decreased in the presence of 1 mg/kg CAY10435 (p<0.05), with a second decrease (p<0.05) observed between 4 mg/kg and 8 mg/kg. Brain tritium levels (○) did not vary upon the addition of 1-12 mg/kg CAY10435 (p>0.05).
Fig. 7 Accumulation of [3H]anandamide and metabolites in the presence of increasing doses of FAAH inhibitors. In the blood, the administration of MAFP (a) resulted in a reduction in [3H]AA (○) (p<0.05) and an increase in [3H]phospholipids (▼) (p<0.05) at every dose examined. [3H]anandamide levels (●) increased (p<0.05) at 8 mg/kg MAFP. A co-administration of CAY10435 (b) resulted in no changes in [3H]anandamide blood levels (p>0.05), but produced reductions of [3H]AA levels (p<0.05) at 4 mg/kg, while increasing (p<0.05) [3H]phospholipid levels only at 12 mg/kg. MAFP produced increases in [3H]anandamide levels in the cerebellum (d) only at 8 mg/kg (p<0.01), with 4 and 8 mg/kg reducing [3H]AA (○) and 8 mg/kg reducing [3H]phospholipid levels (p<0.01). CAY10435 produced no change in the levels of cerebellar [3H]anandamide (p>0.05) (e), but at a dose of 8 mg/kg did produce a slight decrease in [3H]AA (p<0.05) (○) and increase in [3H]phospholipid levels. 12 mg/kg CAY10435 produced a decrease of [3H]phospholipid levels (p<0.05). Effects of FAAH inhibitors upon [3H]anandamide accumulation in FAAH KO mice. Both MAFP and CAY10435 increased nonmetabolized [3H]anandamide in the blood (p<0.05) (c). MAFP produced a slight increase in nonmetabolized [3H]anandamide in FAAH KO blood (p<0.05). In addition, WT MAFP blood samples were not different from all FAAH KO samples (p>0.05). In the cerebellum (f), MAFP-treated WT mice had less [3H]anandamide metabolite accumulation than control animals (p<0.01), yet contained more tritiated metabolites than FAAH KO mice (p<0.01). There was no difference between CAY10435-treated, and untreated cerebellar samples (p>0.05). In addition, no differences in [3H]anandamide or [3H]metabolites were observed among FAAH KO cerebellum samples (p>0.05).
Fig. 8 Comparison of regional accumulation of [3H]anandamide and its metabolites in WT brains with increasing dosage of the FAAH inhibitors (a) MAFP, and (b) CAY10435. a) WT mice co-administered 1-8 mg/kg MAFP + 10 mg/kg anandamide+[3H]anandamide had a heterogeneous accumulation pattern with reductions in tritium levels in 6 of 10 regions in the presence of 1 mg/kg MAFP (p<0.01). Tritium levels in thalamus were reduced with 8 mg/kg MAFP (p<0.01). The periaqeductal gray and hippocampus had no changes in tritium levels (p>0.05). b) Following a co-administration of 1 mg/kg CAY10435 and 10 mg/kg anandamide+[3H]anandamide, 6 of 10 regions exhibited decreases in tritium levels (p<0.01). At doses of 12 mg/kg CAY10435, all regions had reduced tritium levels (p<0.01) except for the periaqeductal gray (p>0.05).

Fig. 9 Comparison of FAAH activity in regional homogenates following MAFP administration. WT mice were i.v. administered 0, 1, or 8 mg/kg MAFP, and their hippocampus, thalamus, and cerebellum isolated 30 min later. Regional homogenates were generated and FAAH activity determined. Following the administration of 1 mg/kg or 8 mg/kg MAFP, anandamide hydrolysis in hippocampal and cerebellar homogenates were reduced (p<0.01) relative to homogenates generated from untreated mice. While 8 mg/kg MAFP was sufficient (p<0.01) to reduce thalamic homogenate FAAH activity, 1 mg/kg MAFP failed to alter anandamide hydrolysis (p>0.5).
Fig. 3

A

% total blood tritium

Time (min)

B

% total brain tritium

Time (min)
Fig. 9

Anandamide hydrolysis (nmol/hour/mg protein)

- **control**
- 1 mg/kg MAFP
- 8 mg/kg MAFP

- hippocampus
- thalamus
- cerebellum