The postmortal accumulation of brain *N*-arachidonylethanolamine (anandamide) is dependent upon fatty acid amide hydrolase activity

Sachin Patel,* Erica J. Carrier,* W-S. Vanessa Ho,* David J. Rademacher,* Sonya Cunningham,* D. Sudarshan Reddy,† J. R. Falck,† Benjamin F. Cravatt,§ and Cecilia J. Hillard[1,*]

Department of Pharmacology and Toxicology,* Medical College of Wisconsin, Milwaukee, WI; Department of Biochemistry and Molecular Genetics,† University of Texas-Southwestern, Dallas, TX; and The Skaggs Institute for Chemical Biology and Departments of Cell Biology and Chemistry,§ The Scripps Research Institute, La Jolla, CA

Abstract ***N*-Arachidonylethanolamine (AEA) accumulates during brain injury and postmortem. Because fatty acid amide hydrolase (FAAH) regulates brain AEA content, the purpose of this study was to determine its role in the postmortal accumulation of AEA using FAAH null mice. As expected, AEA content in immediately frozen brain tissue was significantly greater in FAAH-deficient (FAAH$^{-/-}$) than in wild-type mice. However, AEA content was significantly lower in brains from FAAH$^{-/-}$ mice at 5 and 24 h postmortem. Similarly, wild-type mice treated in vivo with a FAAH inhibitor (URB532) had significantly lower brain AEA content 24 h postmortem compared with controls. These data indicate that FAAH contributes significantly to the postmortal accumulation of AEA. In contrast, the accumulations of two other *N*-acylethanolamines, *N*-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA), were not reduced at 24 h postmortem in either the FAAH$^{-/-}$ mice or mice treated with URB532. FAAH$^{-/-}$ mice accumulated significantly less ethanolamine at 24 h postmortem compared with wild-type mice, suggesting that FAAH activity plays a role in the accumulation of ethanolamine postmortem. These data demonstrate that FAAH activity differentially affects AEA and OEA/PEA contents postmortem and suggest that AEA formation specifically occurs via an ethanolamine-dependent route postmortem.**—Patel, S., E. J. Carrier, W-S. V. Ho, D. J. Rademacher, S. Cunningham, D. S. Reddy, J. R. Falck, B. F. Cravatt, and C. J. Hillard. **The postmortal accumulation of brain *N*-arachidonylethanolamine (anandamide) is dependent upon fatty acid amide hydrolase activity. *J. Lipid Res.* 2005.** 46: **342–349.**

Supplementary key words *N*-acylethanolamines • stroke • ischemia • cannabinoid • endocannabinoid

The endocannabinoid *N*-arachidonylethanolamine (AEA) is a member of the lipid family of *N*-acylethanolamines (NAEs). NAEs and their phospholipid precursors, *N*-acyl-phosphatidylethanolamines (NAPEs), have been shown to accumulate rapidly in the brain postmortem (1, 2). Brain AEA content is also increased after excitotoxic and traumatic brain injury (3), which has led to the suggestion that the formation of AEA and the other NAEs is related to neuronal injury or death. Indeed, increased NAEs, including AEA, were measured in microdialysates from the infarct of a patient with hemispheric stroke (4).

The synthetic enzyme for NAPE, *N*-acyltransferase (NAT), is intracellular and activated by millimolar concentrations of calcium (5). Because both excitotoxicity and loss of membrane integrity during necrotic cell death would result in exposure of NAT to calcium in this concentration range, it has been suggested that the accumulation of NAPE postmortem and during excitotoxicity results from increased NAT activity. NAPEs are hydrolyzed to their respective NAEs via a phospholipase type D (PLD), and the relative amounts of NAEs synthesized generally reflect the *N*-acyl distribution among the NAPEs (6, 7). These data support the hypothesis that NAE production postmortem is a consequence of the calcium-dependent production of NAPEs.

However, there are data in the literature that indicate that NAPE is not the only source of AEA. First, Kempe and colleagues (8) have reported that postmortem rat brain contains measurable amounts of AEA, but they were un-

Manuscript received 30 September 2004 and in revised form 17 November 2004.

Published, JLR Papers in Press, December 1, 2004.
DOI 10.1194/jlr.M400377-JLR200

Abbreviations: AEA, *N*-arachidonylethanolamine; 2-AG, 2-arachidonylglycerol; FAAH, fatty acid amide hydrolase; LC-APCI-MS, atmospheric pressure, chemical ionization liquid chromatography/mass spectrometry; LC-ES-MS, liquid chromatography-electrospray ionization-mass spectrometry; NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; NAT, *N*-acyltransferase; OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine; PLD, phospholipase type D.

[1] To whom correspondence should be addressed.
e-mail: chillard@mcw.edu

able to detect *N*-arachidonylphosphatidylethanolamine, its precursor via the NAT route. Second, the temporal pattern of AEA accumulation in the brain postmortem is different from that of more abundant NAEs such as *N*-palmitoylethanolamine (PEA) (2). In particular, AEA accumulation exhibits a lag phase, whereas the accumulation of the other NAEs is more rapid at early time points. Third, Schmid, Schwab, and colleagues (9) found that, in a rat model of stroke, the NAEs increased 30-fold in the area of the infarct while NAPEs increased only 2.5-fold. All of these studies suggest that an alternative to the NAT/PLD mechanism participates in the increase of AEA during severe ischemia. One possibility is that AEA brain content postmortem increases as a result of a combination of increased synthesis and decreased degradation.

A primary mechanism of degradation of the NAEs is fatty acid amide hydrolase (FAAH) (10). FAAH is an important regulator of endocannabinoid signaling (11); genetic and pharmacological inhibition of FAAH activity results in increased brain AEA content and increased CB_1 receptor-mediated antinociception, for example (11, 12). Given the prominent role of FAAH in the degradation of AEA, we tested the hypothesis that inhibition of FAAH-mediated catabolism of AEA contributes to its accumulation postmortem. Contrary to this hypothesis, we report here that both pharmacological and genetic inhibition of FAAH activity profoundly reduced postmortal accumulation of AEA. However, the postmortal accumulation of two other NAEs, PEA and *N*-oleoylethanolamine (OEA), increases in FAAH null mice. These data indicate that the role of FAAH in the regulation of NAE content of ischemic brain tissue is different for AEA compared with PEA and OEA and suggest that an alternative route for AEA synthesis occurs postmortem that is dependent upon FAAH activity.

MATERIALS AND METHODS

Drugs and animals

FAAH-deficient ($FAAH^{-/-}$) and wild-type C57Bl6 mice of either gender and male ICR mice were used in these studies. All mice were between 18 and 25 g. The $FAAH^{-/-}$ mice were descendants of a previously characterized line and were genotyped as described (11). All mice were housed in cages of five on a 12/12 hour light/dark cycle with lights on at 6:00 AM and had ad libitum access to food and water. All experiments were carried out in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals.

URB532 was synthesized as described previously (12). Ethanolamine and all other buffers and salts were purchased from Sigma-Aldrich (St. Louis, MO). URB597 and deuterated standards were obtained from Cayman Chemical (Ann Arbor, MI). URB532 and URB597 were administered to animals using one of two vehicle preparations: DMSO (50 μl/injection) or in an emulphor vehicle consisting of a ratio of 1:1:8 for drug in DMSO-emulphor-saline (13) using a volume of 300 μl/injection. Controls received an equivalent injection of the vehicle without drug.

For analysis of postmortem NAE and 2-arachidonylglycerol (2-AG) accumulation, mice were killed by rapid decapitation and brains removed. Whole brains were frozen on dry ice immediately, 0.5, 2.5, 5, or 24 h after death. Between the time of death and freezing, brains were kept at room temperature.

Determination of NAE content

Whole brain lipids were extracted as described previously (14). Briefly, brains were weighed and homogenized in acetonitrile containing 34 pmol of $[^2H_8]$AEA and 66 pmol of $[^2H_8]$2-AG. In one experiment, 34 pmol of $[^2H_4]$PEA was also added to the homogenates. The homogenates were sonicated in a cooled bath for 30 min and then incubated overnight at −20°C to precipitate the proteins. Particulates were removed from acetonitrile by centrifugation, the solvent was dried, and extracted lipids were resuspended in methanol. NAEs and 2-AG were quantified in the samples using isotope dilution, atmospheric pressure, chemical ionization liquid chromatography/mass spectrometry (LC-APCI-MS) as described previously with slight modification (14). Samples (5 μl) were separated on a reverse-phase C18 column (Kromasil, 250 × 2 mm, 5 μm diameter) using mobile phase A (deionized water, 1 mM ammonium acetate, and 0.005% acetic acid) and mobile phase B (methanol, 1 mM ammonium acetate, and 0.005% acetic acid). Samples were eluted at 300 μl/min using a linear gradient of 85% solvent B to 90% solvent B over 15 min. Selective ion monitoring, made in the positive ion mode, was used to detect $[^2H_8]$AEA (*m/z* 356), AEA (*m/z* 348), PEA (*m/z* 300), and OEA (*m/z* 326). In one experiment, AEA content was also determined using liquid chromatography-electrospray ionization-mass spectrometry (LC-ES-MS). Samples (5 μl) were separated on a reverse-phase C18 column using mobile phase C (deionized water and 0.005% acetic acid) and mobile phase D (acetonitrile and 0.005% acetic acid). Samples were eluted at 200 μl/min using a linear gradient of 85% solvent D to 100% solvent D over 5 min, and detection was made in the positive ion mode.

In vitro assays of AEA hydrolysis and synthesis

A crude membrane fraction was prepared from the brains either immediately after death or after 24 h at room temperature following the procedure described previously (15). Hydrolysis of AEA was determined as described previously (16) using $[^{14}C]$AEA labeled in the ethanolamine portion of the molecule. The synthesis of AEA from $[^{14}C]$ethanolamine was determined in the same membrane preparation using modifications to a previously published method (17). Membranes (0.1 mg/ml) were incubated in Tris buffer (50 mM, pH 7.4 or 9.0, containing 1 mg/ml BSA) with 36 mM $[^{14}C]$ethanolamine (specific activity 0.25 Ci/mol) and 200 μM arachidonic acid at 30°C. The reaction was stopped by the addition of chloroform-methanol (1:2), and the amount of radioactivity was determined in the aqueous and organic phases. Control incubations were carried out without tissue.

Assay for brain ethanolamine

The amount of free ethanolamine in brain tissue was determined using a previously published procedure (18). Brains were harvested and homogenized in Krebs-Ringer-bicarbonate buffer either immediately or after 24 h at room temperature. The homogenates were diluted to 125 mg protein/ml and added to 0.5 ml of ice-cold trichloroacetic acid (50%, w/v). The extract was filtered, washed, and applied to a column (6 × 0.5 cm) of Dowex 1 × 8 (OH^-) anion-exchange resin. The effluent and water washes were collected until the effluent reached pH 7. These were combined, brought to pH 4 with HCl, and dried under vacuum. Ethanol-soluble material was separated using paper chromatography with butan-1-ol-acetic acid-water (100:25:50). The spots were visualized using ninhydrin spray, and the spots that comigrated with ethanolamine standard were cut out and added to a solution of cadmium chloride (0.01%, w/v) in methanol-

water (60:40) with frequent shaking. After 30 min, the absorption was determined at 510 nm and the ethanolamine concentration was calculated by linear regression using a standard curve.

RESULTS

The recent development of FAAH$^{-/-}$ mice has enabled investigation of the role of FAAH in endocannabinoid signaling and in the metabolism of NAEs (11, 19). In qualitative agreement with a previous report (11), whole brain AEA, OEA, and PEA contents were 5-, 4-, and 7-fold greater, respectively, in FAAH$^{-/-}$ mice compared with wild-type mice immediately after death (**Table 1**). The content of a second endocannabinoid, 2-AG, was not different between the genotypes. Treatment of the mice 1 h before death with URB597, an irreversible inhibitor of FAAH (12), resulted in a significant increase of all three NAEs but did not affect 2-AG content (Table 1).

The effects of postdecapitative ischemia on AEA and 2-AG content were determined (**Fig. 1**). Whole brain AEA content was significantly increased postmortem in wild-type mice. The data shown are the results of the determination of brain AEA content using LC-APCI-MS as described in Materials and Methods; the same accumulation of AEA was seen using the LC-ES-MS protocol, which uses both different chromatography and a different ionization method (data not shown). As has been shown previously (2), the accumulation of AEA exhibited a time delay of several hours before increasing rapidly. Brain AEA content in FAAH$^{-/-}$ mice also increased postmortem; however, the total accumulation at 24 h was only 10% that of wild-type mice (Fig. 1A). 2-AG content was reduced for the first several hours postmortem, then recovered toward the value obtained in fresh brain at 24 h (Fig. 1B). There was no significant difference in 2-AG content between the wild-type and FAAH$^{-/-}$ mice at any time point.

To ensure that the changes in postmortem AEA content were attributable to differences in FAAH activity rather than other genetic differences, we determined the effects of pharmacological inhibition of FAAH on postmortal accumulation of AEA (**Fig. 2**). In this study, the covalent FAAH inhibitor URB532 was administered to male ICR mice at a dose of 10 mg/kg ip 2 h before killing (12), and whole brain AEA content was determined in brains extracted 24 h postmortem. Treatment of the mice in vivo with URB532 significantly reduced postmortem AEA accumulation to ~60% of that determined in vehicle-treated mice.

These data suggest that FAAH is active in brain tissue that has been completely ischemic for 24 h. Indeed, membranes prepared from brains held at room temperature for 24 h after death exhibited robust hydrolysis of AEA (**Fig. 3A**). There was no difference between the maximal activity (Y_{max}) values obtained in membranes freshly isolated compared with those isolated after 24 h at room temperature (33 ± 0.7% vs. 34 ± 1.6%, respectively). However, the rate constant determined under these experimental conditions was significantly lower in the 24 h membranes (0.18 ± 0.02 min^{-1} for control vs. 0.08 ± 0.009 min^{-1} for 24 h; $P < 0.05$ by unpaired *t*-test). No hydrolytic activity was seen in the membranes from FAAH$^{-/-}$ mice either freshly isolated (data not shown) or after 24 h at room temperature (Fig. 3A). These data support the conclusion that AEA hydrolysis in the wild-type brains occurs via FAAH and that FAAH is active as a hydrolase at least 24 h after complete removal of the brain from perfusion.

FAAH has been demonstrated to catalyze AEA synthesis from arachidonic acid and ethanolamine, with a reported K_m for ethanolamine of at least 36 mM (20). If the synthetase activity of FAAH was enhanced relative to its hydrolase function in postmortal brain, this could explain the dependence of the postmortal accumulation of AEA on the presence of FAAH protein. However, we were unable to demonstrate any synthesis of lipid-soluble products from radiolabeled ethanolamine and arachidonic acid upon incubation with either fresh or postmortem tissue (Fig. 3B).

The effects of FAAH deletion on the postmortem accumulation of two other NAEs (OEA and PEA) were determined in a second set of brains. After killing, the brains were held at room temperature for 24 h before lipid extraction (**Table 2**). As above, AEA content was significantly reduced in brain from FAAH$^{-/-}$ mice compared with wild-type mice. However, neither PEA nor OEA content of the same brains was reduced in the FAAH$^{-/-}$ mice com-

TABLE 1. Brain contents of AEA, OEA, and PEA but not 2-AG are increased in FAAH$^{-/-}$ mice and mice treated with FAAH inhibitor extracted immediately after death

Genotype or Treatment	AEA	OEA	PEA	2-AG
		pmol/g brain weight		*nmol/g brain weight*
C57Bl6 wild type	30 ± 4 (6)	345 (1)	552 ± 74 (4)	9.4 ± 0.7 (2)
FAAH$^{-/-}$	148 ± 6 (6)[a]	1,258 (1)	3,658 ± 333 (3)[a]	8.7 ± 0.4 (3)
Vehicle; ICR mice	13 ± 1 (4)	210 ± 14 (4)	235 ± 21 (4)	3.4 ± 0.5 (4)
URB597 (1 mg/kg)	18 ± 1 (4)[a]	720 ± 55 (4)[a]	1,840 ± 241 (4)[a]	3.5 ± 0.2 (4)

AEA, *N*-arachidonylethanolamine; 2-AG, 2-arachidonylglycerol; FAAH, fatty acid amide hydrolase; OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine. Whole brain content of lipids was determined in brains frozen within 2 min of death. Data shown are means ± SEM. Numbers in parentheses refer to the number of brains analyzed. URB597 or vehicle (in two mice the vehicle was DMSO, and in two mice the vehicle was emulphor/saline) was injected into male ICR mice 1 h before death. There was no difference in any lipid measured between the two vehicles, so the data have been combined.

[a] $P < 0.05$ compared with vehicle or wild-type treated mice.

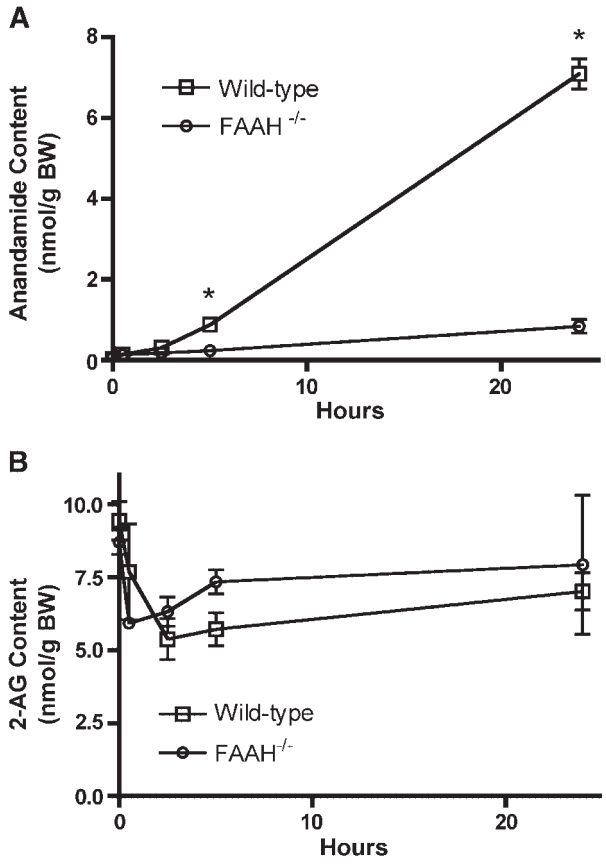

Fig. 1. *N*-Arachidonylethanolamine (AEA) accumulation in wild-type and fatty acid amide hydrolase-deficient ($FAAH^{-/-}$) mice after decapitation. The effects of postdecapitative ischemia on whole brain AEA (A) and 2-arachidonylglycerol (2-AG; B) content (n = 4–6/group) were determined. For AEA accumulation, two-way ANOVA revealed significant effects of both postdecapitative time ($P < 0.0001$) and genotype ($P < 0.0001$) as well as a significant interaction ($P < 0.0001$). Post hoc Bonferroni's test indicated a significant increase in AEA content at 24 h compared with freshly extracted brains in both wild-type and $FAAH^{-/-}$ mice ($P < 0.001$ for both). Pairwise comparisons at each time point using Tukey's post hoc tests indicate that the accumulation of AEA was significantly different between the two genotypes at 5 and 24 h after decapitation. Regarding 2-AG accumulation, two-way ANOVA indicated no significant effect of either genotype or time on the response. BW, brain weight. Error bars represent SEM.

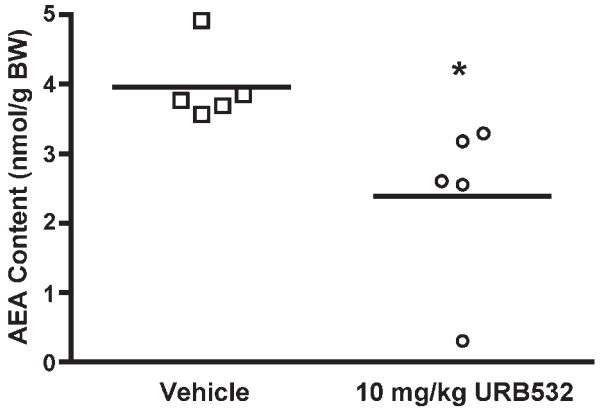

Fig. 2. Treatment in vivo with the irreversible FAAH inhibitor URB532 inhibits postdecapitative brain AEA accumulation. Wild-type mice were treated with vehicle or URB532 (10 mg/kg ip) 2 h before killing (n = 5/group). Brain lipids were extracted 24 h postmortem, and AEA content was determined using LC/MS. * Significantly different from vehicle pretreatment ($P < 0.05$) using an unpaired *t*-test. BW, brain weight.

pared with wild-type mice; in fact, OEA content was significantly increased in the $FAAH^{-/-}$ mice.

We also examined the effects of treatment with URB532 on the postmortem accumulation of AEA, OEA, and PEA. ICR mice were injected with 10 mg/kg URB532 at 2 h before death, and lipids were harvested from the brains 24 h after death. In agreement with our first study, in which AEA alone was measured (Fig. 2), the postmortal accumulation of AEA was significantly reduced in the URB532-treated animals compared with vehicle-treated controls (Table 2). However, brain contents of OEA and PEA were not different at 24 h postmortem between the vehicle-treated and URB532-treated mice.

To determine whether the inhibition of FAAH after death was sufficient to alter the accumulation of AEA, URB597 was added to and mixed with the brains after removal from the mice. After the 24 h postmortem period, the brains were extracted and NAEs measured. When mixed with the brain after death, URB597 had no effect on AEA accumulation, significantly increased the accumulation of OEA, and produced an increase in PEA accumulation that did not reach statistical significance (**Table 3**).

Kempe and colleagues (8) suggested several years ago that postmortal accumulation of AEA could occur via non-enzymatic, base-catalyzed aminolysis of phospholipid acyl chains by ethanolamine. Because one product of FAAH hydrolysis of NAEs is ethanolamine, we explored the hypothesis that the formation of AEA postmortem occurs as a result of increased ethanolamine content in brain and that FAAH-mediated hydrolysis of NAEs is the source of the ethanolamine. Ethanolamine content was determined in brain extracts from wild-type and $FAAH^{-/-}$ mice immediately after killing and after 24 h at room temperature (**Fig. 4**). In agreement with earlier studies (18), the ethanolamine content in wild-type mouse brain was increased 15-fold at 24 h after decapitation. In contrast, although the ethanolamine content from $FAAH^{-/-}$ mice increased slightly during the 24 h after decapitation, this increase did not reach statistical significance. These results suggest that FAAH activity plays a significant role in the generation of ethanolamine postmortem.

DISCUSSION

In qualitative agreement with previous data from the same mouse line (21), we found that brain contents of three NAEs in tissue that was assayed within a few minutes of decapitation were increased 5- to 7-fold in $FAAH^{-/-}$ mice compared with wild-type mice. As expected, the absence of FAAH had no effect on brain 2-AG content. In

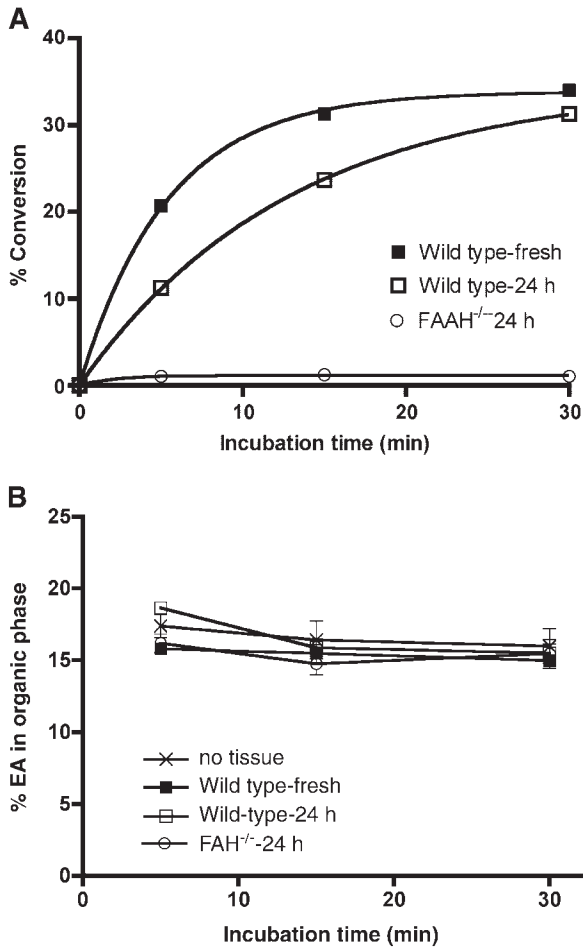

Fig. 3. FAAH hydrolysis is intact at 24 h postmortem, and no AEA synthetase activity is apparent in mouse brain membranes. Brains were harvested from wild-type or FAAH$^{-/-}$ mice, and membranes were prepared either immediately after death or after 24 h. A: FAAH hydrolytic activity was measured in membranes (0.025 mg protein/ml) using the conversion of [^{14}C]AEA to [^{14}C]ethanolamine at various incubation times. B: Putative AEA synthetase activity was determined in membranes (0.1 mg protein/ml) incubated with 36 mM [^{14}C]ethanolamine (EA) and 200 μM arachidonic acid at 30°C. Error bars represent SEM.

addition, when the mice were treated in vivo with an irreversible FAAH inhibitor (12), the NAEs were increased significantly without a change in 2-AG content. Interestingly, comparison of the NAE contents in the control groups for these two studies revealed that the C57Bl6 mice have higher NAE contents than the ICR mice treated with DMSO. At this point, we do not know the reason for this difference. Possible explanations include differences in gender, strain, and exposure to an injection. These data are all in agreement with conclusions from several laboratories that the activity of FAAH plays an important role in the regulation of NAE content in the brain via catabolic removal (11, 12, 14). When FAAH activity is reduced or eliminated, the NAEs accumulate, presumably because their synthesis is unchanged but catabolism is decreased.

However, the current findings demonstrate that the role of FAAH is different when the NAEs are allowed to accumulate postmortem. In the case of two relatively abundant NAEs (OEA and PEA), the absence of FAAH results in a significant increase in their accumulation compared with wild-type mice at 24 h postmortem. The fold difference between the two genotypes was much less at 24 h postmortem than in freshly harvested brain, although the absolute differences were much greater. Irreversible inhibition of FAAH with members of the URB family of carbamates also had a much greater impact on the accumulation of OEA and PEA in freshly isolated brain than after 24 h. These data suggest that the catabolic "efficacy" of FAAH is less at 24 h postmortem than in freshly isolated brains. In other words, the large accumulation of the NAEs at 24 h so overwhelms FAAH-mediated removal that inhibition of FAAH has a lesser effect on content. In any event, the primary role of FAAH in the accumulation of OEA and PEA postmortem is as a route of catabolism.

On the other hand, FAAH appears to play a synthetic role in the postmortal accumulation of AEA, because the FAAH$^{-/-}$ mice accumulated only 10% the amount of AEA as wild-type mice. Because in vivo administration of a FAAH inhibitor also results in significantly lower postmortal AEA accumulation, we conclude that it is FAAH activity that is required for AEA accumulation. We have begun to explore the mechanism by which FAAH activity results in increased brain AEA content postmortem. One possible mechanism is FAAH-mediated synthesis of AEA from arachidonic acid and ethanolamine. Several investigators have demonstrated that recombinant FAAH protein is ca-

TABLE 2. Differential effects of FAAH deletion and inhibition on NAE content 24 h postmortem

Genotype or Treatment	AEA	OEA	PEA
	nmol/g brain weight		
+/+	3.1 ± 0.2	3.3 ± 0.2	7.9 ± 1.3
−/−	0.7 ± 0.1[a]	6.3 ± 0.7[a]	15.0 ± 1.4[a]
Vehicle	9.3 ± 0.8	6.7 ± 0.7	13.6 ± 1.6
URB532 (10 mg/kg)	5.5 ± 0.7[a]	6.5 ± 0.7	14.0 ± 1.5

NAE, *N*-acylethanolamine. Whole brain content of lipids was determined in brains that were left at room temperature for 24 h after removal from the mice. Data shown are means ± SEM. All three lipids were determined in each of six brains. Data were compared using unpaired *t*-tests.

[a] $P < 0.05$ compared with the same lipid content in brain from wild-type or vehicle-treated mice.

TABLE 3. Effects of FAAH inhibition in vitro on the accumulation of NAEs in postmortem brain

Treatment	AEA	OEA	PEA
	nmol/g brain weight		
DMSO	1.1 ± 0.1	4.2 ± 0.5	16.1 ± 1.9
URB597 (1 μM)	1.0 ± 0.08	7.8 ± 1.2[a]	24.8 ± 5.3

Brains were homogenized with either 10 μl of DMSO or URB597 in DMSO and were incubated for 24 h at room temperature before extraction and assay for NAE content. Each value is the mean of four determinations ± SEM.

[a] $P < 0.05$ compared with DMSO-treated brain using an unpaired *t*-test.

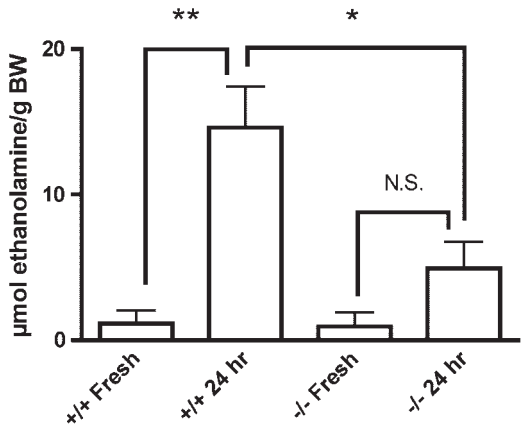

Fig. 4. FAAH$^{-/-}$ mice accumulate significantly less ethanolamine 24 h after decapitation than do wild-type mice. Brains were harvested from wild-type and FAAH$^{-/-}$ mice and were homogenized either immediately or 24 h after harvest (n = 4/group). Whole brain ethanolamine content was determined spectrophotometrically as described in Materials and Methods. One-way ANOVA revealed a significant effect of genotype ($P < 0.001$). * Significantly different ($P < 0.05$) using Bonferroni's post hoc analysis. ** Significantly different ($P < 0.01$) using Bonferroni's post hoc analysis. BW, brain weight. Error bars represent SEM.

pable of catalyzing the reverse of the hydrolase reaction [i.e., is an AEA synthetase if the concentration of ethanolamine is very high (>100 mM)] (17, 22). However, in contrast to earlier studies (23, 24), we did not detect protein-dependent formation of AEA from exogenous ethanolamine and arachidonic acid in membranes from mouse brain. However, the same membrane preparations exhibited robust hydrolysis of AEA at protein concentrations half that used for the synthetase assay. Although it is possible that we would have detected AEA synthesis if we had used higher protein or substrate concentrations, it is our conclusion from these studies that the synthetase activity of FAAH is not likely to contribute to the postmortal accumulation of AEA. However, we cannot discount the possibility that local concentrations of ethanolamine could be high enough to support FAAH operating as a synthetase.

An alternative hypothesis, suggested in 1964 (25) and reconsidered more recently by Kempe and colleagues (8), is that the synthesis of AEA in ischemic brain occurs via a nonenzymatic process of aminolysis of tissue phospholipids and/or triglycerides by ethanolamine. Aminolysis of phospholipid and triglycerides is a well-known phenomenon and can occur under mildly basic conditions (26). As brain homogenates became slightly more alkaline at 24 h after death (data not shown), we considered the hypothesis that the accumulation of AEA postmortem is attributable to aminolysis. The reduction of AEA accumulation postmortem in FAAH$^{-/-}$ mice suggests that the conditions for aminolysis are less favorable in mice of this genotype, perhaps because of reduced ethanolamine content in the FAAH null mice. Indeed, wild-type mice accumulate significantly more ethanolamine at 24 h postmortem than do FAAH$^{-/-}$ mice. The lower amount of ethanolamine in the FAAH$^{-/-}$ mice postmortem would explain a reduction in AEA accumulation at 24 h after death if a large portion of the AEA produced comes from aminolysis of esterified arachidonic acid by ethanolamine. There is no reason to suspect that the second reactant of the aminolysis pathway, arachidonic acid esterified in phospholipid or triglyceride, would be altered by the loss of FAAH, but this possibility remains unexplored at present. We also cannot rule out an effect of the loss of FAAH on other enzymes, such as PLD, which also generate ethanolamine.

Although this mechanism is consistent with our AEA accumulation data, base-catalyzed aminolysis is nonenzymatic, so it is difficult to envision why it would be involved so predominantly in the postmortem accumulation of AEA but not in the accumulation of either OEA or PEA. Base-catalyzed aminolysis involves attack by the amine at the ester bond between the fatty acyl chain and the glyceride backbone. Our data suggest that some unknown factor makes ethanolaminolysis of arachidonate-containing lipids more likely to occur than aminolysis of palmitate- and oleate-containing lipids. Perhaps the answer lies in the localization of the sites of ethanolamine accumulation and membrane regions that are enriched in arachidonate-containing phospholipids. Alternatively, because arachidonate is almost exclusively at the *sn*2 position but palmitate and oleate are more abundant at the *sn*1 position, perhaps the selectivity for the formation of AEA via this route is related to the differential distribution of acyl groups in phospholipids.

It is our current hypothesis that postdecapitative ischemia results in membrane damage, excitotoxicity, and increases in intracellular calcium. As a result, the NAT pathway is activated, resulting in an increase in the synthesis of the family of NAPEs. Subsequent PLD activity yields NAEs, which can either accumulate or be hydrolyzed by FAAH to yield ethanolamine and fatty acids. We further hypothesize that, in addition to the NAT/PLD route, AEA synthesis in the postmortem brain occurs via aminolysis of arachidonate-rich phospholipid or triglyceride by ethanolamine. In the absence of FAAH, the contribution from aminolysis is less because postmortem ethanolamine accumulation is diminished. It is our hypothesis that ethanolamine is shuttled by this mechanism from the total pool of NAEs to AEA through the sequential steps of FAAH-mediated hydrolysis of NAE to ethanolaminolysis-mediated synthesis of AEA. The production of AEA by the aminolysis route overwhelms its hydrolysis by FAAH, at least at 24 h. Presumably, a state steady would eventually be reached between FAAH-mediated hydrolysis of the accumulating AEA and AEA production by ethanolaminolysis.

These data appear to explain several discrepancies in the literature. First, the accumulation of AEA postmortem is out of proportion to the accumulation of its precursor, *N*-arachidonylphosphatidylethanolamine (8, 9). In particular, more AEA is formed than is accounted for by its precursor. Our data are consistent with this observation, because the production of AEA via aminolysis does not involve NAPE. These data could also explain the kinetics of AEA accumulation, which exhibits a lag phase com-

pared with the other NAEs (2), as the accumulation of ethanolamine would be time dependent.

Our results also indicate that FAAH activity plays a role in the postmortem accumulation of ethanolamine: FAAH converts NAEs to ethanolamine and fatty acid. However, the product-substrate relationship between ethanolamine and NAE is not completely clear from these data. When FAAH is deleted, an ethanolamine "deficit" of ~7 μmol/g brain weight is seen at 24 h postmortem. In contrast, our data and those of Schmid et al. (2, 27), who assayed a larger number of NAEs in postmortem mammalian brain, indicate that, at best, the gain in NAE-esterified ethanolamine in a FAAH null brain is only 0.06 μmol/g brain weight. Therefore, the loss of ethanolamine is much greater than the accumulation of NAE, suggesting that the loss of FAAH directly or indirectly alters the production of NAE, perhaps via the NAT/PLD route.

Although these studies were carried out using postdecapitative ischemia as a stimulus for AEA production, aminolysis could also account for some of accumulation of AEA during shorter periods of ischemia. There are several reports in the literature that AEA content increases in intact but ischemic brain (4, 9). AEA content also increases in response to excitotoxic and traumatic neuronal injury (3, 28). Future studies will focus on the relative contributions of the NAT pathway and the FAAH-dependent aminolysis pathway in the synthesis of AEA during shorter periods of brain ischemia. Because CB_1 receptor activity during ischemia modifies the outcome of ischemic and traumatic brain injury (29, 30), it is possible that modulators of FAAH could affect AEA-mediated neuroprotective mechanisms and thus could serve as a novel therapeutic target for the treatment of ischemic brain injury and stroke.

This work was supported by U.S. Public Health Service Grants DA-09155 and GM-31278 (J.R.F.). S.P. was supported by Grant F30 DA-15575 and D.J.R. by Grant F32 DA-16510.

REFERENCES

1. Natarajan, V., P. Schmid, and H. Schmid. 1986. N-Acylethanolamine phospholipid metabolism in normal and ischemic rat brain. *Biochim. Biophys. Acta.* **878:** 32–41.
2. Schmid, P. C., R. J. Krebsbach, S. R. Perry, T. M. Dettmer, J. L. Maasson, and H. H. Schmid. 1995. Occurrence and postmortem generation of anandamide and other long-chain N-acylethanolamines in mammalian brain. *FEBS Lett.* **375:** 143–147.
3. Hansen, H. H., P. C. Schmid, P. Bittigau, I. Lastres-Becker, F. Berrendero, J. Manzanares, C. Ikonomidou, H. H. Schmid, J. J. Fernandez-Ruiz, and H. S. Hansen. 2001. Anandamide, but not 2-arachidonoylglycerol, accumulates during in vivo neurodegeneration. *J. Neurochem.* **78:** 1415–1427.
4. Schabitz, W. R., A. Giuffrida, C. Berger, A. Aschoff, M. Schwaninger, S. Schwab, and D. Piomelli. 2002. Release of fatty acid amides in a patient with hemispheric stroke: a microdialysis study. *Stroke.* **33:** 2112–2114.
5. Hansen, H. S., L. Lauritzen, B. Moesgaard, A. M. Strand, and H. H. Hansen. 1998. Formation of N-acyl-phosphatidylethanolamines and N-acetylethanolamines: proposed role in neurotoxicity. *Biochem. Pharmacol.* **55:** 719–725.
6. Hansen, H. S., L. Lauritzen, A. M. Strand, B. Moesgaard, and A. Frandsen. 1995. Glutamate stimulates the formation of N-acylphosphatidylethanolamine and N-acylethanolamine in cortical neurons in culture. *J. Biol. Chem.* **270:** 23823–23827.
7. Hansen, H. S., L. Lauritzen, A. M. Strand, A. M. Vinggaard, A. Frandsen, and A. Schousboe. 1997. Characterization of glutamate-induced formation of N- acylphosphatidylethanolamine and N-acylethanolamine in cultured neocortical neurons. *J. Neurochem.* **69:** 753–761.
8. Kempe, K., F. F. Hsu, A. Bohrer, and J. Turk. 1996. Isotope dilution mass spectrometric measurements indicate that arachidonylethanolamide, the proposed endogenous ligand of the cannabinoid receptor, accumulates in rat brain tissue post mortem but is contained at low levels in or is absent from fresh tissue. *J. Biol. Chem.* **271:** 17287–17295.
9. Berger, C., P. C. Schmid, W. R. Schabitz, M. Wolf, S. Schwab, and H. H. O. Schmid. 2004. Massive accumulation of N-acylethanolamines after stroke. Cell signalling in acute cerebral ischemia? *J. Neurochem.* **88:** 1159–1167.
10. Schmid, P., M. Zuzarte-Augustin, and H. Schmid. 1985. Properties of rat liver N-acylethanolamine amidohydrolase. *J. Biol. Chem.* **260:** 14145–14149.
11. Cravatt, B. F., K. Demarest, M. P. Patricelli, M. H. Bracey, D. K. Giang, B. R. Martin, and A. H. Lichtman. 2001. Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc. Natl. Acad. Sci. USA.* **98:** 9371–9376.
12. Kathuria, S., S. Gaetani, D. Fegley, F. Valino, A. Duranti, A. Tontini, M. Mor, G. Tarzia, G. La Rana, A. Calignano, A. Giustino, M. Tattoli, M. Palmery, V. Cuomo, and D. Piomelli. 2003. Modulation of anxiety through blockade of anandamide hydrolysis. *Nat. Med.* **9:** 76–81.
13. Cradock, J. C., J. P. Davignon, C. L. Litterst, and A. M. Guarino. 1973. An intravenous formulation of 9-tetrahydrocannabinol using a non-ionic surfactant. *J. Pharm. Pharmacol.* **25:** 345–349.
14. Patel, S., E. R. Wohlfeil, D. J. Rademacher, E. J. Carrier, L. J. Perry, A. Kundu, J. R. Falck, K. Nithipatikom, W. B. Campbell, and C. J. Hillard. 2003. The general anesthetic propofol increases brain N-arachidonylethanolamine (anandamide) content and inhibits fatty acid amide hydrolase. *Br. J. Pharmacol.* **139:** 1005–1013.
15. Hillard, C. J., D. M. Wilkison, W. S. Edgemond, and W. B. Campbell. 1995. Characterization of the kinetics and distribution of N-arachidonylethanolamine (anandamide) hydrolysis by rat brain. *Biochim. Biophys. Acta.* **1257:** 249–256.
16. Jarrahian, A., S. Manna, W. S. Edgemond, W. B. Campbell, and C. J. Hillard. 2000. Structure-activity relationships among N-arachidonylethanolamine (anandamide) head group analogues for the anandamide transporter. *J. Neurochem.* **74:** 2597–2606.
17. Kurahashi, Y., N. Ueda, H. Suzuki, M. Suzuki, and S. Yamamoto. 1997. Reversible hydrolysis and synthesis of anandamide demonstrated by recombinant rat fatty-acid amide hydrolase. *Biochem. Biophys. Res. Commun.* **237:** 512–515.
18. Spanner, S., and G. B. Ansell. 1978. The determination of free ethanolamine in brain tissue and its release on incubation. *J. Neurochem.* **30:** 497–498.
19. Lichtman, A. H., E. G. Hawkins, G. Griffin, and B. F. Cravatt. 2002. Pharmacological activity of fatty acid amides is regulated, but not mediated, by fatty acid amide hydrolase in vivo. *J. Pharmacol. Exp. Ther.* **302:** 73–79.
20. Katayama, K., N. Ueda, I. Katoh, and S. Yamamoto. 1999. Equilibrium in the hydrolysis and synthesis of cannabimimetic anandamide demonstrated by a purified enzyme. *Biochim. Biophys. Acta.* **1440:** 205–214.
21. Clement, A. B., E. G. Hawkins, A. H. Lichtman, and B. F. Cravatt. 2003. Increased seizure susceptibility and proconvulsant activity of anandamide in mice lacking fatty acid amide hydrolase. *J. Neurosci.* **23:** 3916–3923.
22. Arreaza, G., W. Devane, R. L. Omeir, G. Sajnani, J. Kunz, B. Cravatt, and D. Deutsch. 1997. The cloned rat hydrolytic enzyme responsible for the breakdown of anandamide also catalyzes its formation via the condensation of arachidonic acid and ethanolamine. *Neurosci. Lett.* **234:** 59–62.
23. Devane, W. A., and J. Axelrod. 1994. Enzymatic synthesis of anandamide, an endogenous ligand for the cannabinoid receptor, by brain membranes. *Proc. Natl. Acad. Sci. USA.* **91:** 6698–6701.
24. Sugiura, T., S. Kondo, A. Sukagawa, T. Tonegawa, S. Nakane, A. Yamashita, Y. Ishima, and K. Waku. 1996. Transacylase-mediated and phosphodiesterase-mediated synthesis of N-arachidonoylethanol-

amine, an endogenous cannabinoid-receptor ligand, in rat brain microsomes. Comparison with synthesis from free arachidonic acid and ethanolamine. *Eur. J. Biochem.* **240:** 53–62.
25. Wren, J. J., and D. S. Holub. 1964. N-Acylethanolamine lipids: the question of artifact. *Biochem. J.* **90:** 3P–4P.
26. Wren, J. J., and D. S. Merryfield. 1965. Precursors of N-acylethanolamines in hen's egg-yolk lipid. *Biochim. Biophys. Acta.* **98:** 589–597.
27. Schmid, P. C., S. R. Krebsbach, S. R. Perry, T. M. Dettmer, J. L. Maasson, and H. H. O. Schmid. 1996. Corrigendum to: Occurrence and postmortem generation of anandamide and other long chain N-acylethanolamines in mammalian brain. *FEBS Lett.* **382:** 125–126.
28. Hansen, H. H., C. Ikonomidou, P. Bittigau, S. H. Hansen, and H. S. Hansen. 2001. Accumulation of the anandamide precursor and other N-acylethanolamine phospholipids in infant rat models of in vivo necrotic and apoptotic neuronal death. *J. Neurochem.* **76:** 39–46.
29. Panikashvili, D., C. Simeonidou, S. Ben-Shabat, L. Hanus, A. Breuer, R. Mechoulam, and E. Shohami. 2001. An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature.* **413:** 527–531.
30. Mauler, F., V. Hinz, K. H. Augstein, M. Fassbender, and E. Horvath. 2003. Neuroprotective and brain edema-reducing efficacy of the novel cannabinoid receptor agonist BAY 38-7271. *Brain Res.* **989:** 99–111.