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Quantitative LC–MS/MS analysis of arachidonoyl amino acids in mouse brain with treatment of FAAH inhibitor

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Abstract

An additional class of endogenous lipid amides, *N*-arachidonoyl amino acids (Ara-AAs), is growing in significance in the field of endocannabinoids. The development, validation, and application of a sensitive and selective method to simultaneously monitor and quantify the level of Ara-AAs along with anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) in mouse brain has been established. The linearity of the method over the concentration ranges of 0.2–120 pg/μl for the standards of *N*-arachidonoyl amino acids, *N*-arachidonoyl alanine (NAAla), serine (NASer), γ-aminobutyric acid (NAGABA), and glycine (NAGly); 0.7–90 pg/μl for AEA-d₀/d₈; and 7.5–950 pg/μl for 2-AG was determined with *R*² values of 0.99. Also the effects of the FAAH inhibitor URB 597 on the endogenous levels of these analytes were investigated. AEA and NASer brain levels exhibit a dose-dependent increase after systemic administration of URB 597, whereas NAGly and NAGABA were significantly decreased after treatment. NAAla and 2-AG were not altered after URB 597 treatment. The potential benefit of establishing this assay extends beyond the quantification of the Ara-AAs along with AEA and 2-AG in mouse brain, to reveal a variety of pharmacological effects and physiological roles of these analytes.

Keywords

Arachidonoyl amino acids; Anandamide; 2-Arachidonoyl glycerol; FAAH inhibitor; Brain; LC–MS/MS

Anandamide (*N*-arachidonylethanolamide; AEA)¹ and 2-arachidonoyl glycerol (2-AG) are examples of several fatty acid-derived signaling molecules, termed endogenous cannabinoids, that play a modulatory role in neurotransmission. The stimuli as well as the molecular mechanisms responsible for endocannabinoid biosynthesis, release, and metabolism continue to be explored [1–3]. Following the initial discovery of AEA, studies revealed that stimulation of mammalian brain neurons appears to produce a family of *N*-

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¹Abbreviations used: AEA, *N*-arachidonoyl ethanolamide (anandamide); 2-AG, 2-arachidonoyl glycerol; Ara-AA, arachidonoyl amino acids; NAAla, *N*-arachidonoyl alanine; NASer, *N*-arachidonoyl serine; NAGABA, *N*-arachidonoyl-γ-aminobutyric acid; NAGly, *N*-arachidonoyl glycine; AEA-d₈, deuterated anandamide; NAGly-d₈, deuterated *N*-arachidonoyl glycine; FAAH, fatty acid amide hydrolase; APCI, atmospheric-pressure chemical ionization; ESI, electrospray ionization; MRM, multiple-reaction monitoring; LLOQ, lower limit of quantitation; LOD, limit of detection; IS, internal standard; SPE, solid-phase extraction.

acylethanolamides coreleased with AEA [4,5]. There are many different potential endocannabinoid molecules that may be synthesized and released from neurons and other cells. An additional class of related compounds is the long-chain fatty acid amino acid and neurotransmitter conjugates (in our case, the Ara-AAs), which include *N*-arachidonoyl alanine (NAA_{Ala}), serine (NASer), γ -aminobutyric acid (NAGABA), and glycine (NAGly) [6–8]. These compounds appear to have a variety of pharmacological effects, including altering transient receptor potential vanilloid 1 receptors, glycine transporters, orphan G-protein-coupled receptors, and T-type calcium channels [9–13]. The full physiological roles of these compounds, however, have yet to be fully understood, although modulatory roles in the nervous system, vasculature, and immune system are all implicated [9,14].

Biosynthetic and degradation pathways for Ara-AAs have not been fully elucidated, although several conjugation pathways have been implicated in the formation of these molecules. A potential role for the enzyme fatty acid amide hydrolase (FAAH), the primary enzyme responsible for AEA metabolism, has been postulated for the formation of some of the Ara-AAs [15,16]. The possible involvement of FAAH in regulating Ara-AAs is of great significance as FAAH inhibitors are now making their way toward preclinical trials for neuropsychiatric disorders [17,18]. Thus, inhibition of FAAH may alter not only AEA levels but also levels of Ara-AAs as well. We speculate that part of the overall pharmacologic effect of FAAH inhibitors in mammalian systems may be due to effects on Ara-AAs. Our studies are designed to explore this hypothesis further and reveal important new information regarding these neuromodulatory lipids.

Since the first identification of endogenous NAGly, NAGABA, and NAA_{Ala} in rat brain [6] and NASer in bovine brain [13], only a few studies have been reported to simultaneously monitor and quantify the level of Ara-AAs in a physiologically or pharmacologically relevant animal model [14,16,19]. Recently, a targeted lipidomics method based on LC–MS/MS with multiple-reaction monitoring (MRM) and information-dependent acquisition mode was developed to identify the endogenous acyl amino acids in rat [19]. However, the quantitation of NAA_{Ala}, NASer, NAGABA, and NAGly as well as the biosynthetic relationship to AEA metabolism were not addressed. In the current study, we used the FAAH inhibitor URB 597 to probe the regulation of biosynthesis of a variety of Ara-AAs in the mouse brain. Therefore, we report the development of a quantitative method using an LC–MS/MS approach with MRM mode to detect and quantify the level of several important Ara-AAs along with AEA and 2-AG in mouse brain.

Materials and methods

Chemicals and reagents

All the standards of the analytes were obtained from Cayman Chemical (Ann Arbor, MI, USA). Ammonium acetate (>99.99% purity) was obtained from Sigma–Aldrich (St. Louis, MO, USA). The LC–MS-grade water and methanol (MeOH) were obtained from ProteoChem (Denver, CO, USA) and Honeywell Burdick & Jackson (VWR). HPLC-grade acetonitrile was obtained from Sigma–Aldrich. Silica solid-phase extraction (SPE) cartridges (100 mg; Discovery DSC-18Lt SPE) were obtained from Sigma–Aldrich. All the samples were dried with nitrogen gas and freshly resuspended with 100 μ l 70:30 (MeOH:H₂O) prior to LC–MS/MS analysis. All the standards were freshly prepared in MeOH and stored in 12 $\times$ 32-mm polypropylene screw neck vials with LectraBond cap and preslit PTFE/silicone septa from Waters (Milford, MA, USA). URB 597 was acquired from Cayman Chemical.

LC–MS/MS conditions

The liquid chromatography system used for analysis was an Agilent 1200 HPLC (Agilent Technologies, Wilmington, DE, USA). The autosampler was kept at 12 °C. Liquid chromatography was performed on a Zorbax SB-CN 2.1 × 100-mm, 3.5- μ m column (Agilent Technologies). Mobile phase A consisted of 10 mM ammonium acetate. Mobile phase B consisted of 100% methanol. Gradient elution was performed at a flow rate of 300 μ l/min. The initial composition of the gradient was 45% B and was held isocratically for the first half-minute. Then the mobile phase B increased to 70% B within 0.5 min. From 1 to 10 min, the gradient ramped linearly from 70% B to 99% B, where it was held for 4 min. At 14.5 min, the gradient returned to initial conditions of 45% B to reequilibrate the column. The total run time was 21 min. Chromatography was optimized to separate the *N*-arachidonoyl amino acids from the AEA and 2-AG with ideal peak shape and better sensitivity along the gradient.

The HPLC system was connected to an Agilent 6460 triple-quadrupole mass spectrometer (Agilent Technologies). Electrospray mass spectrometry in positive mode conditions with source parameter optimization including cone voltage and positioning were optimized for quantification of analytes by maximizing production of the precursor ion with direct infusion of the standard into the mass spectrometer using single MS monitoring. Nebulizer and sheath gas flow rates of 10 and 8 L/min with source and sheath gas temperatures of 325 and 250 °C, respectively, were used. Subsequently, MS/MS product ion spectra were optimized with respect to the fragment voltage and collision energy. The dominant product ion for each compound was selected to monitor in the MRM mode, and the optimization of dwell time was conducted accordingly. Identification and quantification of analytes in biological tissue were confirmed by comparison of precursor and product ion *m/z* values and LC retention times with standards. The peaks of analytes were integrated and quantified using the MassHunter work-station software Quantitative Analysis for QQQ (version B 03.02; Agilent Technologies). Optimized *m/z* values of precursor and product ions, fragment voltages, collision energy, and retention times chosen for each compound are shown in Fig. 1.

Sample preparation

The extraction procedure was adapted from our previously published method and validated with commercially available standards as well as biological application. Briefly, 2 ml of a 1:1 mixture of cold methanol and acetonitrile spiked with internal standard (IS) AEA-d₈ was added to each tissue sample. A mixture of three internal standards (AEA-d₈, 2-AG-d₈, and NAGly-d₈) was also investigated and the recovery of all three ISs was reasonably close (see Results and discussion). The samples were maintained on ice and homogenized using a Polytron homogenizer (Kinematica). The homogenates were centrifuged for 20 min at 14,000*g* at 4 °C. Supernatants were collected and transferred to clean tubes. Then the supernatants were evaporated to dryness under nitrogen and resuspended in 200 μ l of 70:30 H₂O:MeOH. SPE of the resultant solution was carried out using 100-mg silica cartridges (Discovery DSC- 18Lt SPE). The SPE columns were conditioned with 1.0 ml methanol and equilibrated with 1.0 ml water prior to sample loading. After the extract was loaded, 0.5 ml of LC–MS-grade water was used to wash the cartridge and remove salt and other polar materials. The analytes were eluted using 100% MeOH (2 × 0.8 ml), and fractions were evaporated to dryness under nitrogen and stored at –80 °C until analysis. Prior to mass spectrometry analysis, the sample was resuspended in 100 μ l of 70:30 MeOH:H₂O solution containing 0.1 pmol/ μ l NAGly-d₈ (internal standard for instrument variation).

Validation

Stock solutions of four standard *N*-arachidonoyl amino acids (6.0 pmol/ μ l, containing 0.1 pmol/ μ l of NAGly-d₈) were diluted with 70:30 MeOH:H₂O (with 0.1 pmol/ μ l of NAGly-d₈) solution to generate calibration standards of 3×10^{-4} , 6×10^{-4} , 3×10^{-3} , 6×10^{-3} , 3×10^{-2} , 6×10^{-2} , 3×10^{-1} , and 6×10^{-1} pmol/ μ l. Stock solutions of AEA-d₀/d₈ (0.7 pmol/ μ l) and 2-AG (7.0 pmol/ μ l) were diluted in a similar way to generate calibration standards of 4×10^{-5} , 2×10^{-4} , 4×10^{-4} , 2×10^{-3} , 1×10^{-2} , 5×10^{-2} , and 2.5×10^{-1} pmol/ μ l and 4×10^{-4} , 2×10^{-3} , 4×10^{-3} , 2×10^{-2} , 1×10^{-1} , 5×10^{-1} , and 2.5 pmol/ μ l, respectively. Five microliters of each standard was injected and analyzed in triplicate on 3 separate days. The peak areas of each analyte were calculated and correlated with NAGly-d₈ (IS) to determine the slope and regression coefficient of these calibration curves. The deviation from nominal concentrations over three runs was evaluated to be within $\pm 20\%$ at the lower limit of quantitation for the calibration standards.

The efficiency of the sample extraction procedure was determined from mouse brain homogenate by the method of standard additions. Because of the lack of deuterated standards for most of the analytes, the regular standards of analytes were spiked into the extraction solution along with AEA-d₈ (IS). The bulk mouse brain tissue was processed as described above. All analytes except 2-AG were spiked into mouse brain tissue and homogenized with extraction solvent at concentrations of 0.01, 0.1, and 1 pmol/mg. Because of the higher concentrations of 2-AG reported in brain compared with the other analytes [20], the concentrations of 0.1, 1, and 10 pmol/mg were used as the 2-AG standard addition. Recovery was calculated by comparing the concentrations of the extracted analytes to the concentrations of the nonextracted reference samples. To allow accurate calculation of recovery, endogenous levels of analytes were determined and correlations were made with calculations. The recovery of AEA-d₈ (IS) was also determined with a constant level of concentration. To determine intraday ($n = 6$) and interday ($n = 6$ over a period of 3 days) precision and accuracy, the samples prepared for the recovery assay were used for analysis. Precision was calculated from the relative standard deviation (RSD) of the replicates, and accuracy was calculated by comparison of measured levels of spiked analytes with expected concentrations (RSD). An RSD of 20% was considered acceptable for accuracy and precision at the lower limit of quantitation (LLOQ). The limit of detection (LOD) of each compound was defined as the concentration with a signal-to-noise ratio of greater than 3/1 in the analyzed standard.

Ion suppression effects were determined by adding known amounts of three internal standards (AEA-d₈, 2-AG-d₈, and NAGly-d₈) to the solution prepared from the mouse brain extracts using the above procedure ($n = 3$). As a comparison, the same sample was prepared with a standard solution 70:30 MeOH:H₂O. The integrated peak area of each analyzed compound was measured and compared for evaluation of the ion suppression effects.

Treatment of mice with URB 597

Subjects were 25 adult male, alcohol-naïve HAP2 mice from the 39th generation of selection of the HAP2 line. The mouse line was derived from a progenitor population of outbred HS/Ibg mice (Institute of Behavioral Genetics, Boulder, CO, USA) at the Indiana Alcohol Research Center (Indianapolis, IN, USA). This mouse strain was used because it is part of our ongoing studies examining the endocannabinoid system and alcohol-related behaviors. All experimental procedures were approved by the Purdue Animal Care and Use Committee and were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*. URB 597 (Cayman Chemical) was dissolved in a 75% dimethyl sulfoxide solution and administered at doses of 0.3, 1.0, and 3.0 mg/kg in an injection volume of 5 ml/kg [21]. URB 597 was administered 30 min prior to the fear-potentiated

startle (FPS) testing session. The FPS conditioning and testing parameters were chosen based on, and consistent with, our previous work in HAP2 mice [22]. A single injection and a double injection (separated by 24 h) of URB 597 were administered and brains were immediately extracted 1.5 h after injection.

Statistical differences were determined using ANOVA with a Dunnett's test (SAS 9.2 software; Cary, NC, USA) to compare the treatments versus control.

Results and discussion

Method optimization

Many protocols for quantifying endocannabinoids from animal tissues as well as human plasma are described in the literature [20,23–28]. Most of the methods developed are focused on detection and quantitation of the *N*-acylethanolamine and glycerol derivatives. Very few methods have been reported for quantitation of the increasingly significant *N*-arachidonoyl amino acids [6,14,19]. Herein, we report the development of a new method to successfully detect and quantify *N*-arachidonoyl amino acids along with AEA and 2-AG from mouse brain. There were two main challenges to the success of this method, ionization and chromatographic separation of analytes. Both positive and negative ionization with APCI or ESI sources coupled with various mobilephase systems have been explored [20,23,24,26]. To determine the effects of pH on ionization in both modes, an acidic additive (formic acid), a basic additive (ammonium hydroxide), and a neutral additive (ammonium acetate) were studied (Supplementary Figs. S1–S6). Also the type of organic solvent, either acetonitrile or methanol, was tested for the strength of ionization and elution. The best ionization was obtained using an ESI jet stream source coupled with a 10 mM ammonium acetate, pH 7.3, aqueous solution as solvent A and pure methanol as solvent B. To obtain better chromatographic separation, various types of reverse-phase columns with different gradient systems were tested. The best choice was the cyano column with the selected mobile-phase system that gives the best combinations of analyte peak shape and resolution. Under the current conditions, virodhamine (O-AEA) was separated from AEA with 5 min longer retention time (data not shown), probably because of the stronger interaction with the cyano column. This gradient did not separate 2-AG from its isomer 1-AG. Current literature suggests that 1-AG is possibly produced under particular experimental conditions [1,20]. However, it was reported that 1-AG contributed less than 10% of the combined 2-AG/1-AG peak observed in samples of rat brain tissue under these conditions [20,29]. The combined measurement of 1-AG and 2-AG has been used in previously published methods [20,29,30], and we also adopted this approach.

Subsequently, MS/MS product ion spectra were optimized with respect to the fragment voltage and collision energy. The dominant product ion for each compound was selected to monitor in the MRM mode, and the optimization of dwell time was conducted accordingly (Figs. 1 and 2). We selected the particularly strong product ion for the AEA- d_0/d_8 (m/z 62, representing the ethanolamine fragment), in common with previous studies [20,24]. Information about the fragmentation patterns of the amino acid-containing analytes is variable in the literature [6,14,19,23], partially because of the differing ionization modes and solvent systems used. However, under the current conditions, all the amino acid-containing analytes did produce simple, uniform fragmentation patterns with positive-mode ESI. For NAAa, NAGABA, and NAsr, fragmentation of the amide bond between the arachidonoyl acid core and the amino acids yielded strong protonated amino acid products of m/z 90, 86, and 106, respectively. Interestingly, in the case of 2-AG and NAGly, the charge was typically retained on the fatty acid core, giving the product ion m/z 287 (Fig. 2).

Validation

The analytical performance of the current method was comprehensively validated, including linearity, sensitivity, recovery, precision, and accuracy, and a typical extracted ion chromatogram of the optimized method is shown in Fig. 3. The results confirm that the method provides high recoveries of all analytes and sufficient performance to measure these analytes in mouse brain tissue (Table 1). Because of the endogenous nature of the endocannabinoids and related lipid mediators, it is not appropriate to use the tissue extracts as the matrix for preparing calibration standards, since the baseline level would alter the known calibration amounts. Furthermore, we did not observe any evidence for a significant ion suppression effect by comparing the ion intensities of AEA-d₈, 2-AG-d₈, and NAGly-d₈ in extracted matrix or in organic solvent, the difference of which is in the range of 1–3% (data not shown). Therefore, standard calibration curves were freshly constructed by adding a constant amount of deuterium-containing internal standard, NAGly-d₈ (0.1 pmol/μl), to increasing amounts of the corresponding analytes in organic solvent (see Materials and methods). The peak areas of each analyte were calculated and correlated with NAGly-d₈ (IS) to determine the slope and regression coefficient of these calibration curves. The linearity of the method over the concentration range of 0.2–120 pg/μl for the standards of *N*-arachidonoyl amino acids, 0.7–90 pg/μl for AEA-d₀/d₈, and 7.5–950 pg/μl for 2-AG was determined with R^2 values of 0.99 or greater for each analyte and also was reproducible between the batches (Table 1). The LLOQ, the amount of sample required to give a signal-to-noise (S/N) ratio of 10 or greater with a CV ≤ 20%, was determined to be for AEA, 3.5 pg; 2-AG, 38.0 pg; NAGly, 1.9 pg; NASer, 1.9 pg; NAGABA, 1.0 pg; and NAAla, 1.9 pg on column. Similarly, the LOD, the amount of sample required to give an S/N ratio of 3 or better, was determined to be for AEA, 0.5 pg; 2-AG, 7.6 pg; NAGly, 0.5 pg; NASer, 0.5 pg; NAGABA, 0.4 pg; and NAAla, 0.7 pg on column. The previously reported LOD values are quite variable from report to report, because of the variety of analytical methods and samples [20,23,24,31,32]. However, our current method has a positive comparison of sensitivity with the method using HPLC coupled to atmospheric pressure chemical ionization under similar chromatographic conditions [24], lower LOD values for *N*-arachidonoyl amino acids than reported using other methods [14,19,20,23]. All of these advantages successfully allowed us to complete the quantitation of the analytes with low endogenous levels.

Recovery of each analyte from mouse brain homogenate was determined by the method of standard additions (Table 1). Three structurally representative deuterated analogues, AEA-d₈, 2-AG-d₈, and NAGly-d₈, were first investigated for the recovery of ISs. Two milliliters of the extraction solvent spiked with 100 μl of a mixture of three ISs (0.3 pmol/μl) and 30 mg of brain tissue were processed following the standard extraction procedure. The analyzed mean percentage recoveries (±SD) of AEA-d₈, 2-AG-d₈, and NAGly-d₈ were 73.7 ± 2.8, 74.1 ± 7.5, and 74.6 ± 5.1%, respectively. Since the recoveries of the three ISs were quite similar, we decided to use AEA-d₈ as one single IS for the measurement of other endogenous analogues. During the process, three different concentrations of the analyte standards were added to the aliquots of bulk brain tissue accordingly, along with AEA-d₈ (IS). In certain cases, this approach appeared to overestimate recovery, especially at lower concentrations of added d₀-AEA and 2-AG, even though the endogenous levels were calculated and subtracted from added amounts of standards. However, subtracting a high endogenous background from samples with low concentrations of added standards still affected the accuracy of recovery. The overall sample recoveries of arachidonoyl amino acids were consistent at each spiked concentrations, except for NASer (62%) at lower concentration of added standard.

The results in Table 1 show that the method provides precision and accuracy for quantitation of the targeted analytes from a biological matrix. The intrarun and interrun precisions were within an RSD of 1.0–13.1 and 3.3–17.1%, respectively, across all the spiked standards at

three different concentrations. Because of the higher endogenous level of 2-AG, it appeared that the higher deviations of accuracy always occurred in the samples with the added standards at low concentration. The majority of samples had accuracy between 80 and 120% and precision less than 15%, indicating that the method is robust across the range of concentrations used (0.01–10 pmol/mg).

The biosynthesis and regulation of Ara-AAs are only partially understood. In this study, we proposed to employ the FAAH inhibitor URB 597 to probe the regulation of biosynthesis of a variety of Ara-AAs in the mouse brain. We examined the levels of AEA, 2-AG, NAGly, NAGABA, NASer, and NAAla in brains of mice treated with URB 597 in three different doses (0.3, 1.0, and 3.0 mg/kg). Two sets of dose–response experiments were conducted (see Materials and methods). A single injection and a double injection (separated by 24 h) of URB 597 were administered and brains were extracted 1.5 h after injection. All the analytes in mouse brain tissue (cortical hemispheres not including olfactory bulb and cerebellum) were quantified using the validated method (Supplementary Table S1). The concentrations of AEA, 2-AG, NAGly, NAGABA, NASer, and NAAla of the control mice with a single injection of vehicle were 8.5 ± 0.9 pmol/g ($n = 3$), 1.9 ± 0.3 nmol/g ($n = 3$), 13.1 ± 2.1 pmol/g ($n = 3$), 5.3 ± 0.8 pmol/g ($n = 3$), 3.1 ± 0.5 pmol/g ($n = 3$), and 9.7 ± 1.9 pmol/g ($n = 3$), respectively. The AEA and 2-AG values were consistent with the results of other studies on whole mouse brain, in which about 10 pmol/g (AEA) and 2–4 nmol/g (2-AG) were observed [29,33]. Furthermore, the amount of 2-AG found in brain tissue exceeds that of AEA by approximately 150-fold [34,35]. Previous studies reported that the concentration of NAGly in mouse whole brain was about 13 pmol/g [16], and NAGABA levels are generally similar to those of NAGly, although they differ by about 2-fold between brain regions in rats [9,14], which is similar to the values observed here. To our knowledge, we are the first to report the quantitation of NASer and NAAla in mouse brain.

We observed a dose-dependent increase in AEA brain levels after systemic administration of URB 597 (Fig. 4A and D). The set of mice with two injections of URB 597 at the dose 3.0 mg/kg had a higher level of AEA in their brains than the mice with single treatment, suggesting an accumulation effect resulting from the double injections. In contrast with the observed increase in AEA levels after FAAH inhibition, the levels of NAGly (Fig. 4B and E) and NAGABA (Fig. 4C and F) significantly decreased after treatments, even at the dose of 0.3 mg/kg. However, the increase in dose of URB 597 from 0.3 to 3.0 mg/kg did not seem to cause further increases in these other compounds. Surprisingly, URB 597 did not reduce the level of NASer as observed with NAGly and NAGABA. The dose–response effect of URB 597 on NASer levels, however, appeared to have a response trend similar to that observed for AEA, which was moderately increased with dose (Fig. 5A). NAAla and 2-AG levels were not influenced by systematic administration of URB 597 (Fig. 5B and C).

Conclusions

Predominantly, AEA is hydrolyzed by FAAH into AA and ethanolamine [4,36,37]. Therefore, inhibition of FAAH would reduce the breakdown of AEA, which leads to the observed increase in AEA brain levels. In addition to hydrolysis, however, the presence of an arachidonoyl moiety in AEA suggests the possibility that AEA may also be metabolized by a variety of oxygenases that act on arachidonic acid, such as cyclooxygenase-2, as well as 12- and 15-lipoxygenases [36,38,39]. NAGly is the first discovered endogenous *N*-arachidonoyl amino acid with a wide variety of biological activity whose biosynthesis is still not clearly understood [6,9]. Two primary biosynthetic pathways have been proposed: (1) conjugation of arachidonic acid and glycine [6,16,40,41] and (2) oxygenation of AEA via the sequential enzymatic reaction of alcohol dehydrogenase and aldehyde dehydrogenase [16,40,42]. Our data suggest that the endogenous level of NAGly can be regulated through a

FAAH-dependent conjugation biosynthesis pathway, which is consistent with a previous report from Bradshaw et al. [16]. NAGABA was first isolated from bovine brain tissue along with NAGly and NAAla [6] and was found to have moderate antinociceptive effects in a later study [43]. NAGABA levels are generally similar to those of NAGly in rat brain, although they differ about twofold between brain regions [14]. To our knowledge, our study is the first to measure the endogenous level of NAGABA in mouse brain and reveal that the biosynthetic pathway of NAGABA may be shared with NAGly and be regulated through a FAAH-dependent conjugation biosynthesis pathway. Anandamide modulates a wide range of potassium channels, including voltage-dependent, calcium-activated, and two-pore domain channels [12]. To date, only NASer has been reported to mimic any of these effects [10]. Interestingly, among the four targeted Ara-AAs, only NASer levels were increased by the FAAH inhibitor similar to AEA levels. Experiments in tissue extracts, on the other hand, indicate that the FAAH inhibitor URB 597 did not significantly affect the 2-AG brain level, which is consistent with 2-AG not being metabolized by FAAH as a primary pathway for hydrolysis, but utilizing monoacylglycerol lipase as the primary pathway for metabolism.

The development, validation, and application of a sensitive and selective method for the quantitative analysis of AEA, 2-AG, and four other arachidonoyl amino acids in mouse brain tissue has been established. However, in the future, we might develop an LC method with Ag⁺ coordination or chemical modification coupled with GC-MS/MS to improve the sensitivity or chromatographic separation of a wide range of *N*-arachidonoyl amino acids. Also the dose-response effects of the FAAH inhibitor URB 597 on the endogenous levels of these analytes were investigated. AEA brain levels showed a dose-dependent increase after systemic administration of URB 597; NAGly and NAGABA significantly decreased after treatments; NASer appeared to have a response trend similar to that observed for AEA, which was moderately increased with higher doses; and NAAla and 2-AG did not show a clear dose-dependent response after systematic administration of URB 597. The potential benefit of establishing this analysis assay extends beyond the quantification of the Ara-AAs along with AEA and 2-AG in mouse brain. Biosynthetic and degradation pathways for Ara-AAs have not been fully elucidated, although several conjugation pathways have been implicated in the formation of these molecules. A potential role for the enzyme FAAH, the primary enzyme responsible for AEA metabolism, has been postulated for the formation of some of the Ara-AAs [15]. Since FAAH inhibitors are now being investigated in both clinical and preclinical trials, the impact of these drugs on other biologically active lipids such as the Ara-AAs warrants investigation [17,18]. Furthermore, the Ara-AAs may play a pharmacological role in human disease pathologies ranging from anxiety and stress-related disorders to inflammatory immune disease such as multiple sclerosis. To that end, since these test animals were part of a preliminary study on anxiety and were subjected to fear-conditioning and FPS testing, future studies are warranted to examine the effects of such behavioral tests on the Ara-AAs. Thus, we believe that the current work represents a valuable investigative tool to study the role of endocannabinoids in a variety of biological applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ab.2012.09.031>.

Analyte	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Fragment voltage (eV)	Collision energy (eV)
Anandamide	5.88	348.2	62.2	125	14
Arachidonoyl ethanolamide- d_8	5.88	356.3	62.0	125	14
2-Arachidonoyl glycerol	6.04	379.2	287.1	125	14
<i>N</i> -Arachidonoyl glycine	4.76	362.3	287.1	120	10
<i>N</i> -Arachidonoyl alanine	4.84	376.2	90.1	120	10
<i>N</i> -Arachidonoyl γ -aminobutyric acid	4.89	390.3	86.0	120	10
<i>N</i> -Arachidonoyl serine	4.70	392.2	106.0	120	10
<i>N</i> -Arachidonoyl glycine- d_8	4.76	370.3	295.0	120	10

Fig. 1.

Chemical structures of selected analytes and selected precursor and product m/z values with appropriate retention times, fragment voltages, and collision voltages used to identify and quantify analytes, using MRM with LC-MS/MS.

Fig. 2. Product ion mass spectrum of each targeted analyte, with the chosen precursor ion indicated by [M+H]⁺ and the product ion indicated by the ion mass.

Fig. 3.

Representative MRM chromatograms of the analytes detected in mouse brain and corresponding synthetic standards. (A) All analyte standards, 12 pg of each arachidonoyl amino acid, 20 pg of AEA-d₀/d₈, 300 pg of 2-AG, and 50 pg of NAGly-d₈ on column. (B) All analytes measured, including ISs, in a representative control mouse brain. 4 pg of NASer, 11 pg of NAGABA, 11.3 pg of NAAla, 14.5 pg of NAGly, 32 pg of NAGly-d₈, 814.8 pg of 2-AG, 195.8 pg of AEA-d₈, and 10.4 pg of AEA-d₀ on column. Each chromatogram has been normalized to display the peak of maximum intensity within the time window. LLOQ: AEA, 3.5 pg; 2-AG, 38.0 pg; NAGly, 1.9 pg; NASer, 1.9 pg; NAGABA, 1.0 pg; and NAAla, 1.9 pg on column. LOD: AEA, 0.5 pg; 2-AG, 7.6 pg; NAGly, 0.5 pg; NASer, 0.5 pg; NAGABA, 0.4 pg; and NAAla, 0.7 pg on column.

Fig. 4.

Dose response of endogenous levels of AEA, NAGly, and NAGABA in mouse brains with single or double injections of the FAAH inhibitor URB 597 ($n = 3/\text{group}$). (A) ANOVA with Dunnett's test revealed a significant effect of AEA level (** $p < 0.01$) from the animals that received two injections at doses of 1.0 and 3.0 mg/kg, compared to the vehicle control animals. (B and C) ANOVA with Dunnett's test revealed that URB 597 treatments had significant effects on the levels of NAGly and NAGABA (** $p < 0.01$) across all three doses. (D, E, and F) Comparison of represented extracted chromatograms of AEA, NAGly, and NAGABA in mouse brain with treatments of URB 597 across three different doses.

Fig. 5.

Dose response of endogenous levels of NASer, NAA1a, and 2-AG in mouse brains with single treatment of the FAAH inhibitor URB 597 and with two accumulated treatments ($n = 3/\text{group}$). (A) ANOVA with Dunnett's test revealed a significant effect of NASer level ($*p < 0.05$) in the animals that received two injections at a dose of 3.0 mg/kg, compared to the vehicle control animals. (B and C) ANOVA with Dunnett's test revealed that URB 597 treatments had no significant effect on the levels of NAA1a and 2-AG across all three doses.

Table 1

Recovery, precision, and accuracy (RSD%; $n = 6$) of intraday and interday assay of the LC-MS/MS method for the measurement of arachidonoyl conjugates in mouse brain tissue.

Analyte	R ²	Slope ± SD	Conc. (pmol/mg)	Recovery (% ± SD)	Intraday precision	Intraday accuracy	Interday precision	Interday accuracy
Anandamide	0.995	22.98 ± 0.71	1.00	107.0 ± 6.83	6.2	90.0	7.4	82
			0.10	118.4 ± 15.98	9.7	81.8	8.6	74.1
			0.01	171.4 ± 36.0	5.9	72.7	3.3	45.4
2-AG	0.995	3.74 ± 0.14	10.00	97.8 ± 10.5	10.3	96.1	12.7	104.7
			1.00	110.1 ± 17.5	11.3	104.3	7.7	117.5
			0.10	128.6 ± 20.0	13.1	151.4	17.1	137.3
<i>N</i> -arachidonoyl glycine	0.994	6.21 ± 0.06	1.00	92.2 ± 3.3	2.6	91.4	3.7	91.4
			0.10	98.8 ± 6.0	1.8	105.1	7.8	105.1
			0.01	93.4 ± 14.2	4.8	114.2	15.9	125.7
<i>N</i> -arachidonoyl alanine	0.996	1.26 ± 0.02	1.00	89.0 ± 3.6	2.8	89.1	9.8	91.40
			0.10	93.3 ± 7.1	2.8	91.4	9.5	102.80
			0.01	88.3 ± 15.7	5.4	85.7	11.1	119.00
<i>N</i> -arachidonoyl serine	0.995	5.24 ± 0.19	1.00	81.0 ± 4.0	3.2	80.9	7.2	77.1
			0.10	105.6 ± 6.3	1.0	105.7	6.7	91.4
			0.01	62.0 ± 6.0	10.0	62.9	9.8	65.7
<i>N</i> -arachidonoyl GABA	0.996	22.05 ± 0.81	1.00	78.4 ± 2.7	4.5	78.6	8.0	85.0
			0.10	71.6 ± 4.6	2.1	73.1	4.1	78.6
			0.01	93.1 ± 11.2	4.4	94.3	6.3	114.3