Identification of endogenous acyl amino acids based on a targeted lipidomics approach

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Abstract Using a partially purified bovine brain extract, our lab identified three novel endogenous acyl amino acids in mammalian tissues. The presence of numerous amino acids in the body and their ability to form amides with some saturated and unsaturated fatty acids indicated the potential existence of a large number of heretofore unidentified acyl amino acids. Reports of several additional acyl amino acids that activate G-protein coupled receptors (e.g., N-arachidonoyl glycine, N-arachidonoyl serine) and transient receptor potential channels (e.g., N-arachidonoyl dopamine, N-acyl taurines) suggested that some or many novel acyl amino acids could serve as signaling molecules. Here, we used a targeted lipidomics approach including specific enrichment steps, nano-LC/MS/MS, high-throughput screening of the datasets with a potent search algorithm based on fragment ion analysis, and quantification using the multiple reaction monitoring mode in Analyst software to measure the biological levels of acyl amino acids in rat brain. We successfully identified 50 novel endogenous acyl amino acids present at 0.2 to 69 pmol g\(^{-1}\) wet rat brain. —Tan, B., D. K. O’Dell, Y. W. Yu, M. F. Monn, H. V. Hughes, S. Burstein, and J. M. Walker. Identification of endogenous acyl amino acids based on a targeted lipidomics approach. J. Lipid Res. 2010. 51: 112–119.

Supplementary key words nervous system • lipid • mass spectrometry

Conjugates of fatty acids and amino acids, lipoamino acids, have been identified in biological systems for at least 50 years (1), mostly from bacteria and marine organisms. Significant biological activities have been demonstrated by many lipoamino acids, such as N-(3-hydroxydecanoyl)-L-serine (2), acyl homoserine lactone (3), molop lipids (4), and lipstatin (5). Simple lipoamino acids, called acyl amino acids, contain one fatty acid and one amino acid linked by an amide bond. A family of lipids similar to acyl amino acids, containing only one amide bond connecting a fatty acid with dopamine, amine, or ethanolamine, has been identified in mammalian tissues. These have gained prominence with the identification of the endocannabinoid N-arachidonoyl ethanolamide (6). Other similar bioactive lipids include the putative sleep-inducing lipid oleamide (7), the anti-inflammatory peroxisome proliferator-activated receptor-activated receptor (PPAR\(\alpha\)) activator N-palmitoyl ethanolamide (3, 4), and the pro-nociceptive transient receptor potential vanilloid type 1 (TRPV1) channel activators N-arachidonoyl dopamine and N-oleoyl dopamine (8–10).

Earlier we reported the presence of N-arachidonoyl glycine (NAGly) and N-arachidonoyl gamma aminobutyric acid (GABA) in rats (11), which produced anti-inflammatory and anti-nociceptive effects (11–14). Recently NAGly has been identified as an endogenous ligand for the orphan G-protein coupled receptor GPR18 (15). More recent reports of acyl amino acids with biological effects in mammalian tissues include N-arachidonoyl serine, a vaso dilator (16); a series of N-acyl taurines (17) including N-arachidonoyl taurine, a TRPV1, and TRPV4 receptor activator (18); and N-palmitoyl glycine, which inhibits the heat-evoked response of nociceptive neurons in the rat dorsal horn (19). NAGly, N-arachidonoyl GABA, N-arachidonoyl

Abbreviations: DDA, data dependent acquisition; GABA, gamma aminobutyric acid; IDA, information dependent acquisition; MRM, multiple reaction monitoring; NAGly, N-arachidonoyl glycine; NIST, National Institute of Standards and Technology; PPAR, peroxisome proliferator-activated receptor; SI, silica; SPE, solid phase extraction; TRPV, transient receptor potential vanilloid.

1 This work was dedicated to the memory of Dr. J. Michael Walker.
2 To whom correspondence should be addressed.
3 The online version of this article (available at http://www.jlr.org) contains supplementary data.
serine, and N-arachidonoyl taurine also bind to GPR72 at submicromolar concentrations (20). A host of psychopharmacological effects have also been reported for several synthetic acyl amino acids (21–25) although their existence as endogenous molecules is unknown. Collectively, these findings underscore the realization that the brain and other tissues produce several acyl amino acids and highlight the fact that some of them also exhibit significant biological activities.

The observation that NAGly could be formed by an enzymatically-mediated condensation of arachidonic acid and glycine (11) led us to speculate that a large number of acyl amino acids are formed in mammalian tissues (11), a hypothesis that has received support from the identification of several additional acyl amino acids (26). We have already developed a targeted lipidomic strategy that led to identification and quantification of previously identified acyl amino acids with high efficiency and sensitivity using nano-HPLC/MS/MS and information dependent acquisition (IDA) mode from Analyst QS software, automated computer analysis of MS and MS/MS data, and database searching against mass spectra of synthesized analogs (27). Herein, we report identification of 50 novel acyl amino acids from extracts of rat brains or bovine spinal cords using a targeted lipidomics method. The results demonstrate the occurrence of a large family of acyl amino acids in mammals.

MATERIALS AND METHODS

Materials

Chloroform, tetrahydrofuran, triethylamine, dichloromethane, 2-butyl alcohol, HPLC grade methanol, and acetonitrile were purchased from VWR International, Plainview, NY. Mass spectrometry/HPLC grade acetic acid, formic acid, ammonium acetate, amino acid ethyl ester hydrochlorides, amino acid methyl ester hydrochlorides, and LiOH were purchased from Sigma-Aldrich (St. Louis, MO). HPLC water was purified to a quality of ≥18.0 MΩ cm (e.g., Milli-Q system, Millipore, Billerica, MA). All fatty acids were purchased from Nu-Chek Prep (Elysian, MN). NAGly-d₆ was purchased from Cayman Chemical, Ann Arbor, MI.

Tissue samples

Bovine spinal cords were obtained from the local slaughter house. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed in polycarbonate cages (four per cage) with corncob bedding and stainless steel wire tops under standard conditions (22 ± 1°C, 55% relative humidity, 12 h light/dark cycle). Rats were provided with Teclad 7001 Rat Chow (Harlan, Madison, WI) and water ad libitum. Rats (160–190 g) were randomly selected for experiments. All protocols were approved by the Indiana University Institutional Animal Care and Use Committee.

Work flow of the targeted lipidomics approach

Synthesis. Synthesis of N-arachidonoyl GABA and L-serine was first described in 1995 using iso-butylchloroformate and N-methylmorpholine to couple arachidonic acid anhydride with GABA and serine (28). In our experiment, the N-acyl amino acid ethyl esters were synthesized by coupling the amino acid ethyl ester hydrochloride with the corresponding fatty acid followed by hydrolysis of the ethyl esters with LiOH to generate the acids, as reported previously (19). The reactions gave yields of 80%. Congeners of L-phenylalanine, L-methionine, L-tryptophan, GABA, L-glutamic acid, L-histidine, L-alanine, L-tyrosine, L-valine, L-proline, L-leucine, and L-serine, a general structure shown in Scheme 1, were prepared starting with either the methyl or ethyl ester of the amide and using Hunig’s base during the coupling step. In order to circumvent racemization, hydrolysis of N-acetyl serine and threonine methyl esters was carried out with methanol/water/triethylamine (29). N-acetyl serines were also prepared according to the literature procedure (30). N-arachidonoyl L-glutamines were prepared by adding the sodium salt of glutamine to the mixed anhydride formed after mixing ethyl chloroformate with a solution of the fatty acid, as described previously for the synthesis of volicitin (31). Additional details to the synthesis, purification procedure, and characterization of N-acetyl amino acids using NMR, TLC, and melting point were the same as reported previously (19).

Prescreening method. Methanol (20 vols, g mL⁻¹) was added to extract a whole rat brain (Scheme 2) at 4°C. After addition of HPLC-grade water (2.3 vols), the supernatant was loaded onto preconditioned C18 Bond-Elute solid phase extraction (SPE) columns (Varian, Harbor City, CA). Water and 50% methanol/water (2 mL) were added to wash the columns and the target lipids were eluted with 2 mL methanol. The samples (10 μL) were injected into an ESI-triple quadrupole mass spectrometer (API3000, Applied Biosystems/MDS Sciex, Foster City, CA), coupled with a Zorbax eclipse XDB 2.1 × 50 mm C18 column, a pair of Shimadzu 10A/10A autosamplers (Columbia, MD). With a two solvent system (A: 20% methanol/water with 1 mM ammonium acetate, B: 100% methanol with 1 mM ammonium acetate) and a flow rate of 0.2 mL min⁻¹, the gradient program was as follows: 0–1 min, 5% B; 1–3 min, 5–100% B; 3–6 min, 100% B, 6–7 min, 100–5% B. The MS acquisition methods were multiple reaction monitoring (MRM) methods containing the predicted molecular ions of target acyl amino acids and the theoretical amino acid fragment ions because all the synthetic standards were not made while the prescreening study was conducted. Other parameters of MRM methods were optimized using infusion of NAGly.

Identification methods. METHOD 1. We followed the purification procedure reported previously (11, 27). In brief, six fresh male rat brains (Scheme 2) were obtained by decapitation and extracted using a modified folch extract method at 4°C. The organic phase was purified using diethylaminopropyl, C18, and normal phase column preconditioning (Varian, Harbor City, CA). Water and 50% methanol/water (2 ml) were added to wash the columns and the target lipids were eluted with 2 ml methanol. The samples (10 μL) were injected into an ESI-triple quadrupole mass spectrometer, QSTAR™ Pulsar (QSTAR, Applied Biosystems-MDS Sciex) and an ESI nanospray source (New Objective, Woburn,
The HPLC system used two solvent systems (A: 2% methanol/water (1:1), and exam-
ined with MS analysis. The reverse-phase capillary column, the solvent system, and the gradient used in the LTQ-FT are the same as the ones used in Method 1. The LTQ-FT was operated in data dependent acquisition (DDA) positive mode and set up to perform one full MS scan with the FT analyzer and five MS/MS scans with the ion trap. Mass range and the exclusion time used in DDA LTQ-FT were set to be identical for IDA in the QSTAR.

**Quantification method.** The instrumentation, settings, and purification method were identical to the prescreening method except that MRM conditions were optimized using the quantitative optimization module of Analyst software and infusion of synthetic standards into the API 3000 (27). The transition ions used under negative or positive modes are listed in Table 1. HPLC retention times of acyl amino acids were compared with those obtained with synthetic standards. Exact retention times to aid identification were obtained in positive or negative modes and listed in Table 1. NAGly-d4 (10 pmol g⁻¹) was spiked into the rat brain extract as an internal standard.

**Data analysis.** Data from IDA and DDA experiments were analyzed using the IDA Analyzer as described before (27) to select candidate spectra that contained the mass of a theoretical acyl amino acid and one of the putative fragment ions, [M⁺] or [M⁻]H₂O (Scheme 1). Candidate spectra were interpreted using the National Institute of Standards and Technology (NIST) database (Gaithersburg, MD) equipped with the library of reference MS/MS spectra. To build this library, all 111 acyl amino acids synthesized were flow injected individually at a rate of 5–20 µL min⁻¹ using the QSTAR or LTQ-FT. MS/MS spectra were acquired in the positive and negative ion modes at five different collision energies (20, 25, 30, 35, 40 V). All the MS/MS data were averaged and exported to the NIST database to form the library.

The compounds that we identified using synthetic standards and the QSTAR met the following criteria: <50 ppm error for the molecular ion mass, <200 ppm error for the mass of the amino acid fragment ion, a matching fragmentation pattern, and a minimum number of two monitored ions, the precursor ion and one fragment ion that could be assigned to structures based upon the predicted fragmentation of the candidate molecule. Data obtained using the LTQ-FT provided high-accuracy molecular weight determination of the precursor ions whereas the MS/MS spectra were generated in the ion trap with unit mass resolution. The compounds identified in the positive or negative ion modes without the use of standards met the following minimum criteria: <45 ppm error for the theoretical mass of the molecular ion, <200 ppm error for the theoretical mass of the amino acid fragment, and a minimum number of three monitored ions that could be assigned to structures of the candidate molecule.

**RESULTS AND DISCUSSION**

**Prescreening method.**

Our confidence in the presence of acyl amino acids came from the prescreening study. Rat brain methanolic extracts were purified using C18 SPE columns and analyzed by MRM methods using an API3000. We selected the predicted molecular ions and the amino acid fragment ions [M⁺] or [M⁻]H₂O (Scheme 1) as the transition ions for MRM analysis due to their high ion intensities and structural significance. As reported before (27), there were MRM signals corresponding to numerous unknown acyl amino acids in the extract of the rat brains. After prescreening for all acyl amino acids (combination of arachidonic acid, palmitic

**Scheme 2.** Targeted lipidomics strategy for identification and quantification of acyl amino acids.
acid, oleic acid, stearic acid, and docosahexaenic acid and 24 amino acids), we found signals for most of them. We decided to focus our attention on the species with high intensity signals, which were about 70 acyl amino acids.

**TABLE 1. Summary of novel acyl amino acids identified using targeted lipidomics approach**

| Identification method | Parent ion mass error (Da) | Matching fragments | Number of assigned fragments | Identification | Quantification | Retention (min) | prod g⁻¹ wet brain |
|-----------------------|-----------------------------|--------------------|-------------------------------|----------------|----------------|----------------|---------------------|
| N-oleyl alanine       | +14.0                        | 9                  | 4                            | ✓              | 426/88         | 4.22           | 2.4                 |
| N-palmitoyl alanine   | -11.0                        | 7                  | 4                            | ✓              | 354/98         | 4.19           | 5.7                 |
| N-stearoyl alanine    | -11.0                        | 9                  | 4                            | ✓              | 432/102        | 4.13           | 12.7                |
| N-oleoyl glutamic acid| -7                           | 9                  | 4                            | ✓              | 456/102        | 4.12           | 13.5                |
| N-palmitoyl glutamic acid| -10                        | 9                  | 4                            | ✓              | 410/102        | 4.20           | 31.4                |
| N-stearoyl glutamic acid| -9                           | 9                  | 4                            | ✓              | 384/102        | 4.18           | 36.5                |
| N-docosahexaenoyle GABA| -2.5                        | 20                 | 4                            | ✓              | 412/102        | 4.16           | 16.5                |
| N-oleyl GABA          | -1                            | 8                  | 4                            | ✓              | 366/102        | 4.06           | 2.1                 |
| N-palmitoyl GABA      | +1                            | 4                  | 4                            | ✓              | 340/102        | 4.13           | 2.6                 |
| N-stearoyl GABA       | -1                            | 6                  | 4                            | ✓              | 368/102        | 4.06           | 2.5                 |
| N-arachidonoyl glutamic acid| +8.8                        | 20                 | 4                            | ✓              | 431/145        | 3.60           | 2.1                 |
| N-palmitoyl histidine⁴| +28                           | 5                  | 4                            | ✓              | 394/156        | 4.18           | N/A                 |
| N-arachidonoyl (iso)leucine²| +2.0                        | 7                  | 5                            | ✓              | 416/130        | 4.19           | N/A                 |
| N-oleoyl (iso)leucine²| +0.2                           | 4                  | 7                            | ✓              | 394/130        | 4.36           | N/A                 |
| N-palmitoyl (iso)leucine²| +0.5                          | 4                  | 4                            | ✓              | 368/130        | 4.36           | N/A                 |
| N-oleoyl methionine   | +28                           | 7                  | 4                            | ✓              | 412/148        | 4.25           | 1.6                 |
| N-palmitoyl methionine| +36                           | 6                  | 6                            | ✓              | 386/148        | 4.22           | 3.3                 |
| N-stearoyl methionine| +24                            | 6                  | 5                            | ✓              | 414/148        | 4.34           | 2.4                 |
| N-docosahexaenoyle phenylalanine²| +10                        | 5                  | 3                            | ✓              | 474/164        | 4.17           | N/A                 |
| N-oleoyl phenylalanine| +1                             | 5                  | 5                            | ✓              | 428/164        | 4.20           | 1.1                 |
| N-palmitoyl phenylalanine| +3                            | 6                  | 3                            | ✓              | 402/118        | 4.19           | 2.7                 |
| N-stearoyl phenylalanine| +3                            | 6                  | 3                            | ✓              | 430/164        | 4.31           | 1.9                 |
| N-oleoyl proline²| +0.5                           | 4                  | 5                            | ✓              | 378/114        | 4.21           | N/A                 |
| N-palmitoyl proline²| +0.3                           | 4                  | 6                            | ✓              | 352/114        | 4.22           | N/A                 |
| N-stearoyl proline²| +0.5                           | 5                  | 5                            | ✓              | 390/114        | 4.30           | N/A                 |
| N-palmitoyl serine   | -0.2                           | 8                  | 7                            | ✓              | 342/104        | 4.12           | 58.6                |
| N-stearoyl serine    | -46                            | 9                  | 7                            | ✓              | 370/104        | 4.26           | 35.2                |
| N-oleoyl threonine  | +1                             | 4                  | 3                            | ✓              | 382/118        | 4.18           | 5.3                 |
| N-palmitoyl threonine| +0.1                           | 3                  | 5                            | ✓              | 356/118        | 4.15           | 69.0                |
| N-oleoyl tryptophan  | +0.7                           | 6                  | 5                            | ✓              | 467/203        | 4.20           | 0.6                 |
| N-palmitoyl tryptophan| +11                            | 6                  | 4                            | ✓              | 441/203        | 4.18           | 2.1                 |
| N-stearoyl tryptophan| +6                             | 6                  | 5                            | ✓              | 469/203        | 4.29           | 1.9                 |
| N-oleoyl tyrosine    | -3                             | 3                  | 2                            | ✓              | 444/180        | 3.78           | 0.5                 |
| N-palmitoyl tyrosine| -1                             | 3                  | 2                            | ✓              | 418/180        | 4.05           | 0.5                 |
| N-stearoyl tyrosine  | -3                             | 3                  | 2                            | ✓              | 446/180        | 4.14           | 0.2                 |
| N-linoleoyl valine⁷| +5.5                           | 6                  | 5                            | ✓              | 379/116        | 4.07           | N/A                 |
| N-palmitoyl valine²| +4.1                           | 4                  | 4                            | ✓              | 354/116        | 3.95           | N/A                 |
| N-stearoyl valine⁷| +4.0                           | 3                  | 4                            | ✓              | 382/116        | 4.03           | N/A                 |
| N-stearoyl arginine³| +43.8                          | N/A                | 4                            | ✓              | 441/175        | 4.05           | N/A                 |
| N-oleoyl asparagine³| +0.6                           | N/A                | 3                            | ✓              | 397/133        | 4.16           | N/A                 |
| N-docosahexaenoyle glutamine³| +13.1                        | N/A                | 8                            | ✓              | 457/130        | 4.31           | N/A                 |
| N-oleoyl glutamine³| +31.0                          | N/A                | 5                            | ✓              | 411/130        | 4.12           | N/A                 |
| N-palmitoyl glutamine³| +42.5                          | N/A                | 7                            | ✓              | 409/145        | 4.12           | N/A                 |
| N-stearoyl glutamine³| +9.6                           | N/A                | 5                            | ✓              | 413/130        | 4.27           | N/A                 |
| N-arachidonoyl histidine³| +19.4                        | N/A                | 3                            | ✓              | 442/156        | 4.08           | N/A                 |
| N-docosahexaenoyle histidine³| +26.0                        | N/A                | 3                            | ✓              | 466/156        | 4.1           | N/A                 |
| N-oleoyl histidine³| +6.9                           | N/A                | 3                            | ✓              | 420/156        | 4.16           | N/A                 |
| N-linoleoyl taurine⁹| +32.9                          | N/A                | 4                            | ✓              | 388/124        | 4.05           | N/A                 |

¹ Identification is denoted by ✓. ² Some are identified in spinal cord. The levels in brain are hard to determine. ³ These are identified without standards. ⁴ N/A indicates data are not available.

**Identification method**

Identification was based on matching the HPLC retention times and the fragmentation patterns of the constituents in tissues with the synthetic standards. To obtain
information for our targeted acyl amino acids, we divided the identification into several steps: enriching the target lipids, using nano-LC/MS/MS to produce MS/MS spectra with high sensitivity, and analyzing spectra with all possible acyl amino acids using software. After several years' research on NAGly and other acyl amino acids, our laboratory developed a specific purification method based on the chemical structures of acyl amino acids by combining anion exchange, reverse phase, and normal phase SPE to enrich acyl amino acids (11, 27). Without these purification steps, it is impossible to dissolve rat brain extracts in such a small volume (100 µl) with methanol/water mixtures. Our nano-HPLC/MS/MS system has demonstrated the ability to provide good quality MS/MS spectra with 150 femtano-LC/MS/MS system has demonstrated the ability to provide good quality MS/MS spectra with 150 femto-

Nano-HPLC/MS/MS system has demonstrated the ability to provide good quality MS/MS spectra with 150 femtogram-scale resolution in one run due to the computer-aided selection of ions for MS/MS. Therefore, we can obtain MS/MS spectra with high sensitivity and high efficiency after we combined IDA or DDA with nano-LC. For the third step, we designed a computer program known as IDA Analyzer to analyze more than one thousand spectra generated in IDA experiments with the more than one hundred possible acyl amino acids. The algorithm of this program was to search the predicted parent ions and the most abundant and characteristic fragments, the amino acid fragment ions [M_\text{aa}^-] or [M_\text{aa}-\text{H}_2\text{O}^-]. The candidate spectra were imported to the NIST database to be compared with the MS/MS library. In another study, we used this workflow to identify 11 previously identified acyl amino acids with high efficiency and sensitivity (27).

In an attempt to maximize the number of identified novel low-abundance lipids, we employed two different MS instruments, the QSTAR and LTQ-FT. LTQ-FT has high-mass accuracy for the molecular ions but a low-mass cutoff and unit mass resolution for the fragment ions collected in the ion trap. As summarized in Table 1, 15 compounds identified in the LTQ-FT have mass errors of less than 1 ppm, 5 with errors between 1 and 5 ppm, and 5 with errors of 5 and 10 ppm. This accuracy provides greater confidence in our assigned structures. Because of the low-mass cutoff, the MS/MS spectra obtained using the LTQ-FT often lack the most abundant low-mass fragment ions such as the amino acid moiety that can play a vital role for the assignment of structures, e.g., m/z 88 for acyl alanines. We addressed this limitation by employing the QSTAR, which has good mass accuracy and the ability to capture ions down to m/z 50. Following our workflow and using both the QSTAR and LTQ-FT enabled us to identify 39 out of 50 compounds. We used targeted MS/MS scans on the QSTAR for additional 11 lipids.

Acyl amino acids, except 10 without synthetic standards, identified in the tissues had the matching retention times and fragmentation patterns with the synthetic standards. Two typical examples, N-stearoyl GABA and N-palmitoyl glutamic acid, are provided in Fig. 1. Both exhibited almost identical MS/MS spectra for the endogenous lipids and the synthetic standards. Although the presence of these compounds in mammalian tissues was not known until now, they were reported to exhibit a variety of biological effects. N-stearoyl GABA antagonized catalepsy induced by haloperidol at low doses, potentiated sedation induced by apomorphine, and caused antinociception and hypothermia (25, 32–36). N-palmitoyl GABA, N-palmitoyl glutamic acid, and N-stearoyl glutamic acid exhibit similar psychopharmacological effects (25, 32–36). They also have neuroprotective or neurodegenerative effects (25, 32–36). Among the rest of acyl amino acids identified here, N-stearoyl proline, N-stearoyl phenylalanine, N-stearoyl tryptophan, N-stearoyl arginine, and N-stearoyl tyrosine showed antimicrobial activity against bacteria and yeast (37).

**Method 1**

Using Method 1, 14 acyl amino acids were found in a positive IDA experiment of the 55% methanol/chloroform SI SPE fraction of the DEA fraction of 0.5% ammonium acetate, which was the highest number of novel compounds in one single run using Method 1. Analysis of the total of six IDA experiments in positive and negative modes for all three SI SPE fractions (25%, 55%, and 85% methanol/chloroform SI SPE fractions) identified 20 compounds (Table 1).

Analysis of all DEA fractions using MRM scans in the API 3000 showed that N-acyl glutamic acids are present only in the DEA fraction of 5% ammonium acetate/methanol. This fraction was collected, further purified (C18 SPE), and analyzed by targeted MS/MS using the QSTAR. We identified the N-acyl glutamic acids N-arachidonoyl, N-palmitoyl, N-stearoyl, N oleoyl, and N docosahexaenoyl glutamic acid (Table 1).

In the negative mode, all glutamic acid series, such as N-palmitoyl glutamic acid in Fig. 1, fragmented to palmitic anion [RCOO]^- (m/z 255) with high intensity. The majority of acyl amino acids only generated anions such as [RCONH]^− with extremely low intensity, as shown for N-stearoyl tyrosine (supplementary data). The anions [RCOO]^- generated from glutamic acid series are due to the cyclization reaction of glutamic acid side chain. Similar phenomenon occurred to the dipeptide of leucine and aspartic acid in which aspartic acid is on the C-terminal position (37). The mechanism is shown in the supplementary data.

**Method 2**

Using Method 1 we were able to identify 25 acyl amino acids, which is less than half of the total number of acyl amino acids with high intensity in our prescreening result. For the identification of additional target lipids, the purification procedure was modified (Method 2; Scheme 1) to further reduce the sample complexity. Three rat brains generated 20 fractions after SPE and semi-preparative HPLC, compared with only three fractions where most acyl amino acids existed using Method 1. All fractions were then subjected to MRM analysis using the API3000. Fractions that indicated the presence of acyl amino acids were subjected to targeted MS/MS scans in the QSTAR and the LTQ-FT. Fourteen novel acyl amino acids were identified in addition to those identified using Method 1 (Table 1). To test our theory of the existence of acyl amino acids in other nervous
Targeted lipidomics, acyl amino acids, nerve system

After a simple C18 SPE clean up were analyzed by LC/MS/MS API3000 system, which is the same as the prescreening method. The retention times from this study were compared with the synthetic standards, as shown in Fig. 1. All identifi ed acyl amino acids, except the ones without corresponding synthetic standards, have the same retention times as the synthetic standards.

To quantify the concentrations of our target lipids in rat brain extracts, we used NAGly-d₈ as an internal standard to correct for matrix effects because it was the only deuterated acyl amino acid commercially available. The recovery rate was 85% and this was used as the recovery rate for all acyl amino acids. Although we synthesized 111 N-acyl amino acid standards, their high cost precluded synthesis of stable labeled analogs. Our experiments were designed to minimize the postmortem delay. Typically, only two minutes elapsed between decapitation of the rat and the start of centrifugation in chloroform/methanol. Compared with tissues, Method 2 was applied to bovine spinal cord, which led to the identification of 11 acyl amino acids (Table 1).

By this method, we also identified several fatty acid conjugates of leucine or isoleucine. The N-acyl leucine and N-acyl isoleucine pairs had the same retention times, identical m/z of the parent ions, and indistinguishable fragmentations. As a result, the compounds identified in our experiments (Table 1) could be either or both series of compounds. N-Acyl leucine and isoleucines (myristic, palmitic, palmitoleic, oleic acid) were found in Deleya marine (bacteria) (38) and N-arachidonoyl isoleucine was reported to inhibit the hydrolysis of anandamide by fatty acid amide hydrolase at nmol concentrations (39).

**Quantification method**

The API3000 LC/MS/MS system with a flow rate of 0.2 ml min⁻¹ was used to provide information on the retention times and the endogenous concentrations. Samples

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Fig. 1. Chromatogram and MS/MS spectra of N-stearoyl GABA (1a–d) and N-palmitoyl glutamic acid (2a–d) as standards and in rat brain extract. MRM chromatograms of constituents in rat brain extract (1a and 2a) and of synthetic standards (1b and 2b) using quantification method. MS/MS spectra of constituents in rat brain extract (1c) and synthetic standard (1d) in negative mode. MS/MS spectra of constituents in rat brain extract (2c) and synthetic standard (2d). Important peaks are listed in supplementary data.
microwave irradiation, ischemia induced by decapitation was shown to increase the levels of anandamide (40) and arachidonoyl-CoA by 4-fold and to decrease the level of docosahexaenoyl-CoA by 2-fold (41) in rat brains within two to five minutes of decapitation. All the other acyl-CoAs (41) and amino acids had changes of less than 40% (42). Based on the structural similarity of anandamide and possible roles played by acyl-CoAs in biosynthesis of N-acetyl amino acids (explained in the section on biosynthesis and metabolism), decapitation in our method probably caused changes of 2-fold or less within 5 min except that arachidonoyl amino acids could increase 4-fold.

Using this quantification method, we determined rat brain concentrations for 29 of 50 novel acyl amino acids. For some lipids with very low abundance in the rat brain, such as the valine and proline series, bovine spinal cord tissue was used to obtain identification information so the biological levels in the rat brains were not determined. For lipids identified from tissues without corresponding synthetic standards such as most of glutamine and histidine series, information on biological levels could not be obtained. The amount of the compounds varied widely (Table 1), some occurring at low levels (e.g., N-stearoyl serine, 0.2 pmol g⁻¹) and others at much higher levels (e.g., N-palmitoyl threonine, 69 pmol g⁻¹). Among the compounds with the same fatty acid group, the serine and glutamic acid series are the most abundant and the species with the tyrosine moiety are less abundant. The high concentrations of N-acetyl taurine (100–300 pmol/g) (27) and glutamic acid (12–16 pmol/g) may reflect the abundance of taurine and glutamic acid in brains. To confirm this claim, further study of distributions of acyl amino acids in different tissues will need to be done. The high concentrations of N-acetyl serines (35–58 pmol/g) might be due to abundance of N-acetyl phosphatidyl serines (0.1% porcine brain lipids) (43), (explained in the section on biosynthesis and metabolism). Among the compounds with the same amino acid group, those with palmitoyl, stearoyl, and oleoyl groups are more abundant than those with arachidonoyl and docosahexaenoyl groups, which matches the abundance of these fatty acids either as free acids or as phospholipids (44, 45), especially in phosphatidylcholines.

**Biosynthesis and metabolism**

These observations suggested that acyl amino acids may be generated by conjugation of fatty acids and amino acids in biological systems or may be metabolites of the corresponding phospholipids. N-Acetylglutamate synthase, required for the urea cycle in animals and arginine biosynthesis in plants, catalyses the production of N-acetylglutamate from acetyl-CoA and glutamate. Palmitoylcarntine with an O-linked ester bond is generated from N-palmitoyl-CoA and carnitine by carnitine palmitoyl transferase I in the outer membrane of mitochondria. It is possible that an enzyme with functions similar to N-acetyl glutamate and carnitine palmitoyl transferase I can catalyze the formation of N-acetyl amino acids. This theory is supported by reports that an enzyme isolated from rat liver promoted the condensation of both palmitic and oleic acids with phenylalanine (46) and that cytochrome c has shown in vitro to catalyze the formation of N-acetyl glycines and N-arachidonoyl dopamine, serotonin, and GABA (47, 48) in the presence of acyl-CoAs, H₂O₂, and amino acids at pH 7.4.

Analogous to the observation that N-acetyl phosphatidyl ethanolamine can serve as the precursor of N-acetyl ethanolamines such as anandamide (49, 50), N-acetyl serines could be products of N-acetyl phosphatidyl serines, endogenous compounds in brain and other cells (43), either by the hydrolysis reaction with phospholipase D or deacylation with phospholipase A and then hydrolysis with phosphodiesterases. Other biosynthetic routes may exist following on the example of anandamide being oxidized to N-arachidonoyl glycine (51). Degradation of N-acetyl amino acids could be carried out by fatty acid amide hydrolase based on its ability to hydrolyze anandamide (52).

**CONCLUSIONS**

Using a targeted lipidomics approach adapted from proteomics, we identified and quantified 50 novel endogenous acyl amino acids with high sensitivity. Our results are the first comprehensive survey of these compounds in mammalian tissues and support our initial hypothesis that there exists a large family of endogenous acyl amino acids that was based upon observations of the presence of a few endogenous acyl amino acids. These results stress the utility and power of an MS-based targeted lipidomics approach as several low-abundance acyl amino acids were readily identified despite the presence of other more abundant lipids. The strategies developed here should hasten the process of identification and biological characterization of these and many other novel low-abundance signaling lipids in the future.

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