Journal of Chemical Neuroanatomy 55 (2014) 24–37

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Neurochemical study of amino acids in rodent brain structures using an improved gas chromatography–mass spectrometry method

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ARTICLE INFO

Article history:
Received 17 September 2013
Received in revised form 22 November 2013
Accepted 26 November 2013
Available online 7 December 2013

Keywords:
Rodents
Amino acids
Neurochemistry
Gas chromatography–mass spectrometry

ABSTRACT

The analysis of amino acid levels is crucial for neuroscience studies because of the roles of these molecules as neurotransmitters and their influence on behavior. The present study describes the distribution and levels of 16 amino acids (alanine, asparagine, aspartic acid, cysteine, glycine, glutamic acid, isoleucine, leucine, lysine, methionine, phenylalanine, proline, sarcosine, serine, valine, and threonine) in brain tissues (prefrontal cortex, striatum, hippocampus and cerebellum) and the serum. Neurochemical analysis was performed on Wistar rats and C57BL/6 mice using an efficient method for extraction, a fast microwave-assisted derivatization and gas chromatography–mass spectrometry analysis. The amino acid concentration varied across brain regions for 14 of the 16 analyzed molecules, with detection limits ranging from $0.02 \pm 0.005\ \mu mol\ L^{-1}$ to $7.07 \pm 0.05\ \mu mol\ L^{-1}$. In rats, the concentrations of alanine, glycine, methionine, serine and threonine were higher in prefrontal cortex than in other areas, whereas in mice, the concentrations of glutamic acid, leucine and proline were highest in the hippocampus. In conclusion, this study provides a cerebral profile of amino acids in brain regions and the serum of rats and mice.

1. Introduction

The analysis of amino acid levels is crucial for the study of neuroscience because amino acids play important roles as neurotransmitters and can influence behavior under physiological conditions. In neuroscience, the amino acids are divided into non-neurotransmitters, which are important for structural and metabolic functions, and neurotransmitters, which are able to deliver messages across synapses (Shah et al., 2002). Amino acid neurotransmitters provide both excitatory and inhibitory neurotransmission in the central nervous system (CNS). The inhibitory amino acids (IAAs), including alanine, glycine, GABA, and taurine, reduce the activity of post-synaptic neurons. In contrast, the excitatory amino acids (EAAs), including aspartate, cysteine, homocysteine, and glutamate, increase the excitability of post-synaptic neurons (Shah et al., 2002).

Changes in amino acid levels in the CNS are widely associated with psychiatric and neurological disorders. EAAs can damage neurons by excessive stimulation; this phenomenon is called excitotoxicity and is involved in brain ischemia, epilepsy and Alzheimer's disease. Disruptions in glutamate neurotransmission have also been associated with schizophrenia and bipolar disorder. In addition, changes in neurotransmission by IAAs such GABA and glycine are associated with anxiety and some motor disorders (Bowery and Smart, 2006; Rajendra et al., 1997).

Abbreviations: aCSF, artificial cerebrospinal fluid; BSTFA, N,O-bis(trimethylsilyl)-trifluoroacetamide; CNS, central nervous system; EAA, excitatory amino acids; GC, gas chromatography; HPLC, high-performance liquid chromatography; IAA, inhibitory amino acids; LOD, detection limits; LOQ, quantification limits; MS, mass spectrometry; OPA, *o*-phthalaldehyde; MTBSTFA, N-(tert-butyldimethylsilyl)-N-methyltrifluoro-acetamide; TMCS, trimethylchlorosilane.

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http://dx.doi.org/10.1016/j.jchemneu.2013.11.002

The amino acid neurotransmitters can be found at concentrations ranging from nanomolar to micromolar in the CNS, requiring high-performance methods for accurate quantification of these molecules. These methods involve two steps. First, it is necessary to separate the neurotransmitters, which can be done by chromatography. Next, the molecules are detected, which depends on their chemical features. Amino acids are small aliphatic molecules and do not possess chemical features that permit direct analysis, as fluorescent emission or UV absorbance, necessitating an additional stage of derivatization (Shah et al., 2002). For analysis of amino acids, *o*-phthalaldehyde (OPA) is the most common derivatization agent, generating derivatives with fluorescent and electroactive features after reaction with primary amines in the presence of thiol groups. The amino acids derivatized with OPA are usually separated using high-performance liquid chromatography (HPLC) and analyzed with fluorescent detectors. However, there is an alternative method, which uses gas chromatography (GC). In this method, the amino acids are converted into more volatile analytes to be separated by GC, which can be monitored using either electron capture or mass spectrometric (MS) detection (Shah et al., 2002). The GC–MS method for detection of amino acids provides greater selectivity and ability to distinguish analytes, and it is more accurate than HPLC for the measurement of amino acids (Shah et al., 2002).

Because amino acids act as neurotransmitters on different cerebral substrates and in several neuropathologies, it is important to have effective methods for evaluating the profiles of these molecules in health and disease conditions. Therefore, this work describes the application of a recently developed GC–MS method (Paiva et al., 2013) in prefrontal cortex, striatum, hippocampus, cerebellum and serum samples from Wistar rats and C57BL/6 mice.

2. Materials and methods

2.1. Animals

Wistar rats (250–300 g) and C57BL/6 mice (25–30 g) of both sexes were used in this study. The animals were housed in plastic cages, maintained on a 12:12 h light–dark cycle and fed ad libitum. The experimental protocol was approved by the Ethics Committee for Animal Experimentation of our institution (Protocols N°. 042/11 and 182/13).

2.2. Chemicals and solutions

The artificial cerebrospinal fluid (aCSF) was manufactured with chemicals from Sigma–Aldrich (St. Louis, MO, USA) as follows: 127 mmol L^{-1} NaCl, 2 mmol L^{-1} KCl, 1.2 mmol L^{-1} KH_2PO_4, 26 mmol L^{-1} $NaHCO_3$, 2 mmol L^{-1} $MgSO_4$, 2 mmol L^{-1} $CaCl_2$, 10 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 mmol L^{-1} glucose, bubbled with carbogenic mixture (95%, v/v O_2 and 5%, v/v CO_2).

The lysis buffer solution was prepared with a Sigma-FAST Protease Inhibitor cocktail Tablet (St. Louis, MO, USA), which contains 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mM Phosphoramidon, 130 mM Bestatin, 14 mM E-64, 1.0 mM Leupeptin, 0.2 mM Aprotinin, 10 mM Pepstatin A, and 50 mM sodium fluoride, and 1 mM sodium orthovanadate was added. The final pH was 7.20.

The following amino acid standards were obtained from Sigma–Aldrich (St. Louis, MO, USA): L-alanine, L-asparagine, L-aspartic acid, L-cysteine, L-glycine, L-glutamic acid, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, sarcosine, L-serine, L-valine, L-threonine. The standard solution of each amino acid was prepared at an initial concentration of 1.0 mg mL^{-1} in H_2O and stored at −20 °C. Primary standard mixtures, from the separate standard solutions, containing 10 μg mL^{-1} of the indicated amino acids, were prepared in H_2O. These working solutions were stored at 4 °C.

HPLC-grade methanol was obtained from Merck KGaA (Darmstadt, Germany), *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) 99:1 (v/v), and pyridine anhydrous 99.8% (m/m) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and methoxyamine hydrochloride 98% m/m was obtained from Fluka (St. Louis, MO, USA).

2.3. Collecting and sample preparation procedures

The animals were euthanized by decapitation, and blood samples were collected. Next, the brain was rapidly removed from the cranium and submerged in ice-cold aCSF. The regions of interest (prefrontal cortex, striatum, hippocampus and cerebellum) were dissected out on an ice-cold plate as described by Chiu et al. (2007). Then, each component was weighed, placed in a 1.5 mL microcentrifuge tube and homogenized in 400 μL of lysis buffer solution for 30 s. After homogenization, the samples were centrifuged (8000 × *g* for 10 min at 4 °C), and aliquots of the supernatants were stored at −20 °C until the derivatization step. The blood samples were centrifuged to collect the serum (2000 × *g* for 5 min at 4 °C).

2.4. Derivatization procedure

The derivatization procedure was performed as described in Paiva et al. (2013). Briefly, an aliquot of 100 μL of supernatants homogenate was transferred to a tube (or 50.0 μL, for cerebellum and serum), and to precipitate the protein was added 900 μL of methanol at −10 °C for each tube. The solution was vortexed for 1 min and centrifuged at 10,000 rpm for 10 min at room temperature. Then, 100 μL of supernatant was transferred to a glass GC vial and evaporated to dryness at room temperature under N_2. Next, 15 μL of methoxyamine in pyridine at 20 mg mL^{-1} was added, followed by 35 μL of BSTFA with 1% TMCS. The solution was vortexed for 30 s and submitted to microwave irradiation in a domestic microwave oven (700 W power) equipped with a turning table for 3 min. The final solution was used for GC–MS analysis.

2.5. Analysis of gas chromatography–mass spectrometry

The amino acid analyses were performed using a Shimadzu (Kyoto, Japan) GC–MS system model GC-2010/QP-2010 high performance quadrupole. The mass spectrometer operated in electron impact mode (EI) at 70 eV. The analytes were separated on a Restek (Bellefont, PA, USA) Rtx-5MS fused-silica capillary column chemically bonded (30 m × 0.25 mm id × 0.25 μm film thickness) containing 5% diphenyl, 95% dimethylpolysiloxane. The oven temperature program began at 80 °C, rose to 200 °C at 8 °C min^{-1}, rose to 300 °C at 30 °C min^{-1}, and held that temperature for 3 min. The injector was operated at 280 °C in splitless mode for 3 min, followed by a 1:20 split ratio. Helium with a purity of a 99.99% was used as the carrier gas, at a flow rate of 1.0 mL min^{-1}. Samples were injected manually with a 10 μL microsyringe Hamilton (Reno, NV, USA), in splitless mode, with a 1 min vent open time, and an injected volume of 1.0 μL. The ion source temperature was 200 °C and the GC–MS interface was kept on 260 °C. For identification and confirmation, each amino acid solution was prepared separately for derivatization in optimized conditions. Next, 1.0 μL of solution was injected into the GC–MS apparatus, and the analysis was performed in full scan mode (range 45–300 *m*/*z*), with a scan time of 2 scan s^{-1}. The retention times achieved and ions used for