Published in final edited form as:

ACS Chem Neurosci. 2017 June 21; 8(6): 1251–1261. doi:10.1021/acscchemneuro.6b00398.

D-Amino Acid Levels in Perfused Mouse Brain Tissue and Blood: A Comparative Study

Choyce A. Weatherly[†], Siqi Du[†], Curran Parpia[†], Polan T. Santos[‡], Adam L. Hartman^{‡,§}, and Daniel W. Armstrong^{†,*}

[†]Department of Chemistry and Biochemistry, University of Texas at Arlington, Arlington, Texas 76019, United States

[‡]Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, United States

[§]Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205, United States

Abstract

The L-enantiomer is the predominant type of amino acid in all living systems. However, D-amino acids, once thought to be “unnatural”, have been found to be indigenous even in mammalian systems and increasingly appear to be functioning in essential biological and neurological roles. Both D- and L-amino acid levels in the hippocampus, cortex, and blood samples from NIH Swiss mice are reported. Perfused brain tissues were analyzed for the first time, thereby eliminating artifacts due to endogenous blood, and decreased the mouse-to-mouse variability in amino acid levels. Total amino acid levels (L-plus D-enantiomers) in brain tissue are up to 10 times higher than in blood. However, all measured D-amino acid levels in brain tissue are typically ~10 to 2000 times higher than blood levels. There was a 13% reduction in almost all measured D-amino acid levels in the cortex compared to those in the hippocampus. There is an approximate inverse relationship between the prevalence of an amino acid and the percentage of its D-enantiomeric form. Interestingly, glutamic acid, unlike all other amino acids, had no quantifiable level of its D-

*Corresponding Author: sec4dwa@uta.edu. Fax: 817-272-0619.

Present Address

A.L.H. current address: Division of Clinical Research, National Institute of Neurological Disorders and Stroke, Neuroscience Center, Rockville, MD 20852.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchemneuro.6b00398.

Representative chromatograms of the first and second dimension separations of standard Fmoc amino acids, second dimension chiral chromatography conditions for the separation of Fmoc amino acids, representative chromatograms of first and second dimension separations of Fmoc amino acids from samples, and mass spectra and diode array UV peak purity assay of L- and D-Fmoc amino acids from biological samples for method verification (PDF)

ORCID

Daniel W. Armstrong: 0000-0003-0501-6231

Author Contributions

D.W.A. and A.L.H. devised this project. C.A.W., S.D., C.P., and D.W.A. developed analytical methodology, data analysis, and sample derivatization of D-amino acids. P.T.S. assisted in the preparation and dissection of all mice. C.A.W., A.L.H., and D.W.A. contributed to the writing of the manuscript. This article was prepared while A.L.H. was at Johns Hopkins University.

The authors declare no competing financial interest.

antipode. The bioneurological reason for the unique and conspicuous absence/removal of this D-amino acid is yet unknown. However, results suggest that D-glutamate metabolism is likely a unidirectional process and not a cycle, as per the L-glutamate/glutamine cycle. The results suggest that there might be unreported D-amino acid racemases in mammalian brains. The regulation and function of specific other D-amino acids are discussed.

Graphical Abstract

Keywords

D-Amino acid levels; cortex; hippocampus; blood; perfused brain tissue; D-amino acid regulation

1. INTRODUCTION

Amino acids are among the most important molecules in nature. The first discovered amino acid was asparagine, which was isolated from asparagus extract in 1806.¹ Subsequently, the analysis of protein hydrolysates revealed additional analogous compounds that are now referred to as amino acids.² In 1851, Louis Pasteur revealed the optical activity of asparagine and aspartic acid,³ leading to the realization that most common amino acids have optical activity arising from their differing orientations around the α -carbon.⁴ The initial discovery and configurational assignment of amino acids led to the opinion that L-configuration amino acids were solely found in nature and D-amino acids were laboratory artifacts.^{5,6}

Dispelling the notion that D-amino acids are “unnatural” or not biologically relevant began in the mid-20th century with the report that D-amino acids were an integral part of the bacterial peptidoglycan.⁷ It was the first report that D-amino acids, specifically D-alanine and D-glutamic acid, were appurtenant biological entities. Subsequent evidence began to emerge supporting the notion that D-amino acids were not uncommon in living systems. In 1969, J. Corrigan published a review with 30 examples of D-amino acids found in invertebrates.⁵ In some cases, a functional role was implied, whereas in many others, it was unknown. By the end of the last century, with the advent of new bioanalytical techniques, scientists were able to easily isolate and identify D-amino acids in a greater variety of biological samples and, in particular, vertebrates.^{8–12} In 1986, free D-aspartic acid was found in human and animal tissues.¹² Subsequently, free L- and D-amino acids were reported in pathologically relevant human urine, plasma, cerebrospinal fluid, and amniotic fluid.^{8,9} A nonproteinic amino acid, D-pipecolic acid, was found to be an indicator of the severity of a neurological genetic disease.¹³ Additional reports showed that D-amino acid-

containing peptides had distinct functions including binding to specific opiate receptors and acting as neurotoxins blocking voltage-sensitive calcium channels.^{14,15} Since the early 2000s, there have been additional reports of free D-amino acids in various mammalian tissues.^{16–18}

Investigations into the role and function of specific D-amino acids in mammalian systems are intriguing but relatively neoteric. D-Serine was found to be a coagonist of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor, which could occupy the glycine binding site.^{19,20} Free D-serine has been determined to be localized primarily in the mammalian forebrain, where the highest concentrations for NMDA receptors are found.^{21–23} In cases with treatment-resistant depression, D-serine has been used as a potential biomarker of ketamine antidepressant response.²⁴ Recently, D-leucine has been applied as an effective treatment for seizures in mice.²⁵ However, the exact mechanism through which D-leucine acts to inhibit seizure activity remains unknown. D-Serine and D-leucine are two recent and prominent examples of D-amino acids found in brain tissues having specific function or actions. Most D-amino acids are metabolized by either D-amino acid oxidase (DAO) or D-aspartic acid oxidase. It is thought D-amino acid oxidase is important for regulating levels of select D-amino acids and is crucial to help control levels of D-serine.^{19,20}

As evidence accrues on the unique biological roles of D-amino acids, it has become apparent that there are limited fundamental studies on normal, baseline levels of most L- and D-amino acids in the brain and blood of any mammalian entity. The few limited studies may not have had sufficient sensitivity to detect D-amino acids.^{26,27} Hippocampus and cortex regions of the brain are two of the most common anatomical sources of epilepsy.²⁵ Examination of these tissues to determine baseline levels of D-amino acids will aid in establishing essential normative data that further studies can reference in finding functions relating to neurological pathology and potentially other unexplored D-amino acid processes. In this work, we provide the most complete characterization of brain and blood amino acid levels in mice. Furthermore, the levels are examined in terms of anomalies, trends, and possible relevance to the limited existing data on mammalian D-amino acids.

Transcardial perfusion is a standard technique involving vascular perfusion of anesthetized animals for preservation of tissues for analysis.²⁸ It is unclear how much of each amino acid is in the intravascular and extravascular spaces, which could provide useful information about their potential physiological roles. Regarding D-amino acid analyses, perfusion has been applied only to the determination of D-serine and its function in the activity of DAO in the human central nervous system.²⁹ The effect of perfusion in the analysis of a spectrum of L- and D-amino acids in brain tissues has never been examined.

2. RESULTS AND DISCUSSION

Tables 1 and 2 provide the amino acid data for both the cortex and hippocampus of nonperfused and perfused mice, respectively. Table 3 shows both the total and D-amino acid levels in the blood. Both the total amino acid and especially D-amino acid levels are usually lower in the blood (often by an order of magnitude) (Tables 1 and 3 and Figure 1A,B). Figure 1A shows 12 averaged concentration levels of total amino acids (i.e., D- plus L-

amino acids). The three amino acids with the highest levels in brain tissues are glutamic acid, glutamine, and aspartic acid. Figure 1B shows 12 averaged concentration levels of D-amino acids. Concentrations of D-glutamine, D-aspartic acid, and D-serine have the highest values. Interestingly, no detectable amount of D-glutamic acid was found. This was unexpected because the concentration of total glutamic acid is significantly higher than all other amino acids, and all other amino acids detected have significant levels of their D-enantiomers present. Concentrations of D-amino acid levels are almost always lower than the corresponding L-amino acid levels (Figure 1A vs Figure 1B). For example, concentrations of D-glutamine in the nonperfused cortex and concentrations of D-isoleucine in the nonperfused hippocampus are approximately 4 times less than their total amino acid values. Concentrations of D-phenylalanine, D-leucine, D-isoleucine, and D-allo-isoleucine are among the lowest values. Distinct from other amino acids, the ratio of blood to nonperfused cortex or hippocampus levels of branched chain amino acids, particularly leucine, is closer to unity than other amino acids (Figure 1A). However, the level of their D-amino acid antipodes remains elevated in brain tissue compared to blood (Tables 1–3).

Free amino acid concentrations obtained from perfused tissue may yield a more accurate measurement of cellular and extracellular/extravascular levels. Table 2 lists the amino acid values obtained from the perfused tissues, and Figure 2 shows the effect of perfusion on the amino acid levels measured in the cortex and hippocampus. A plot of the average amino acid values of perfused tissue vs nonperfused tissue is linear (see Figure 2). Each data point is an amino acid, and the measure of R^2 (how close the data are fitted to the regression line) represents the uniformity of the effect of perfusion. In the raw data of Figure 2B for the hippocampus tissues and Figure 2D for the cortex tissues, the slope of the line is approximately 0.85. This represents an average 15% decrease in total amino acid levels in both the perfused cortex and hippocampus relative to the nonperfused samples. In terms of reproducibility, mouse-to-mouse variation in total amino acid levels is less for perfused mice (see *y*-axis or ordinate range) than nonperfused mice (see *x*-axis or abscissa range).

The hippocampus tissue values in Figure 2B and cortex tissue values in Figure 2D have a high amount of mouse-to-mouse variation. To reduce this variation, a normalization technique was applied to the data. The hippocampus tissue values in Figure 2A and cortex tissue values in Figure 2C show the normalized data. Glutamic acid, the highest concentration amino acid, was used to normalize the data. Normalization considerably reduces the mouse-to-mouse variation in amino acid values in the perfused and nonperfused tissues (see Figure 2A,C vs Figure 2B,D). However, the average values of all the amino acid levels (defined by the slope of the lines) show no significant change.

Mouse whole brain samples were also analyzed. It was found that the total amino acid levels were within the same range as those reported for cortex and hippocampus tissues. Whole brain D-amino acid levels were 10–70% lower, except for D-aspartic acid, which was similar. This also indicates that total amino acid values do not appear to change due to dissection.

2.1. Broad Trends

2.1.1. Effect of Perfusion—Because of interorganism anatomical variation in vascular anatomy, the presence of blood in brain tissue is an additional variable that affects any study of a blood-based analyte in tissue. As a result, the cerebral blood volume (CBV) varies between mice.³⁰ Perfusion largely corrects this source of interorganism variation, representing a significant technical advance. This technical issue may, in turn, have an impact on the interpretation of the importance of D-amino acids (and other analytes) in normal physiology and disease. Limiting this source of variation also may make the detection of differences between normal and abnormal physiology easier to detect, particularly in the context of subtle findings.

2.1.2. Amino Acid Levels: Homeostasis?—D-Amino acids are introduced into biological systems from food, bacterial sources, and indigenous biological processes.^{8,31,32} As D-amino acids are introduced to biological systems, a variety of processes must be present to regulate D-amino acid levels. Both DAO (see Introduction) and amino acid racemases^{33,34} affect the levels of their substrates in biologically unspecified ways. Other processes also must be operative (*vide infra*). Biological processes must be present to effect control of the highly elevated brain levels of most D-amino acids as well as the anomalously low level of D-glutamic acid therein. Linking specific D-amino acids and their levels to biological function and/or disease is the ultimate challenge.

2.1.3. Cortex vs Hippocampus Amino Acid Levels—A plot of the cortex and hippocampus total amino acid levels is linear with a slope of 1, indicating that there is little difference between the total amino acid levels in the cortex and hippocampus (Figure 3A). In contrast, a similar plot of D-amino acid levels showed a slope of 0.87, indicating that, on average, levels of D-amino acids are 13% lower in the cortex (Figure 3B), which points to the need for further characterization of the effects of D-amino acid function in the hippocampus. The only exception is D-aspartic acid, which is 2 times higher in the cortex. As noted previously, D-aspartic acid has the highest concentration of any D-amino acid in blood, and there is more D-aspartic acid in blood than all other D-amino acids combined.

One process for the control of multiple D-amino acids levels is the expression or activity of DAO.³⁵ DAO oxidizes D-amino acids, except for aspartic acid and glutamic acid, to their corresponding imino acids, producing ammonia and hydrogen peroxide.³⁵ Hydrogen peroxide is a reactive oxygen species associated with oxidative stress. In this article, baseline amino acid levels from healthy wild-type mice exhibited unique regional discrepancies between the hippocampus and cortex tissues. The approximate 13% decrease in D-amino acid levels in the cortex is an indication that there may be regional differences in D-amino acid synthesis and degradation. DAO might be one of the enzymes responsible for this difference. However, immunoblot and immunocytochemical studies in rodent and human tissues have not shown a difference in immunoreactivity or protein levels between hippocampus and cortex.^{36,37} Prior work examining endogenous enzyme activity levels in rodent brain subregions was not sensitive enough to show DAO activity in either cortex or hippocampus, despite DAO there being immunoreactivity and mRNA expression in both areas.³⁸ Nonetheless, differences in enzyme activity between these regions might account for

some of the 13% decrease.³⁹ These findings may also support the hypothesis that additional processes exist other than DAO to control levels of D-amino acids in brain tissues. In fact, it has been shown that formation of D-serine from L-serine, via a serine racemase enzyme, is one process that both synthesizes and degrades D-serine in rat brain.³³

In contrast to most of the other D-amino acids, the outlier D-aspartic acid (see Figure 3B) is not oxidized by DAO but, rather, by D-aspartate oxidase.^{40,41} D-Aspartic acid has a 2 times higher concentration in cortex tissues (Figure 3B). This suggests the activity (or expression) of D-aspartate oxidase is less in cortex tissues. It was found that D-aspartate oxidase, visualized by enzyme histochemistry, was present in cortex and hippocampus tissues of rats.⁴⁰ However, D-aspartate oxidase was almost 2 times more concentrated in the hippocampus.⁴⁰ Other processes may contribute to D-aspartic acid metabolism in the cortex relative to the hippocampus.

2.1.4. Percent D-Amino Acid Levels—Figure 4 shows plots of % D-amino acids versus total amino acids in the hippocampus and cortex. Such plots of % D-amino acid levels allow for a deeper analysis of our findings and detection of interesting relationships and possible anomalies. These plots indicate that there is an approximate inverse relationship between the prevalence of an amino acid and its percent D-enantiomeric form. With the exception of allo-isoleucine in the hippocampus tissues, all % D-amino acid levels are similar between the two tissues. There are at least two additional highly interesting features revealed in these data. First, glutamic acid, the most prevalent of all free amino acids, had no quantifiable level of its D-antipode, the only amino acid where this was noted. This may reflect D-glutamic acid metabolism, and the biological relevance of this finding is interesting but unknown. Second, the least prevalent of the measured amino acids, allo-isoleucine, was found to have approximately equal amounts of it D- and L-antipodes in the hippocampus (discussed below).

High % D-serine was observed in both brain regions (Figure 4). As noted previously, D-serine plays a role in NMDA-type glutamate receptor function.^{19,20} DAO affects only D-serine, but D-serine racemase, a reversible enzyme, affects levels of both L and D-isomers of serine.^{33,37,42,43} Formation of D-serine from L-serine, via serine racemase, is an important mechanism for maintaining the levels of D-serine in rat brain.³³ Ratios of D- to L-amino acid interconversion rates (reported as % D-amino acid values) may be an indication of racemase activity. Currently, serine and aspartate racemases are the only D-amino acid racemases reportedly found in mammalian brains.^{33,34} Our results suggest that there might be other D-amino acid racemases in mammalian brains that cause the high % D-amino acid values in Figure 4 (e.g., phenylalanine, asparagine, isoleucine, etc.). This does not exclude the probability that other biological processes affect levels of L- and D-amino acids.

2.2. Specific Amino Acids

2.2.1. D-Glutamic Acid (Glutamate) and D-Glutamine—At physiological pH, L- and D-glutamic acid exist as their carboxylate anions, L- and D-glutamate. L-Glutamate is the most abundant and principal excitatory neurotransmitter in the brain.⁴⁴ Given its importance, it is not surprising that L-glutamate has a well-known regulatory pathway, the glutamate–

glutamine cycle.⁴⁴ In this cycle, L-glutamate is supplied to the central nervous system from L-glutamine.^{45,46} Astrocytes convert L-glutamate to L-glutamine via glutamine synthetase.⁴⁵ L-Glutamine is then released into the extracellular space.⁴⁵ Conversely, L-glutamine is metabolized into L-glutamate in presynaptic terminals by the mitochondrial enzyme glutaminase.⁴⁵ The levels of L-glutamate and L-glutamine found in the hippocampus and cortex of NIH Swiss mice were not only higher than any of the other amino acids, but they were also an order of magnitude higher than the blood levels (Figure 1A). Only serine had comparably elevated levels in brain tissues relative to blood levels. More surprisingly, D-glutamic acid was the only D-amino acid not found (i.e., below quantitation limits) in either the brain or blood. Conversely, D-glutamine was the most prevalent D-amino acid in the cortex, with hippocampus levels being about 10 times lower and blood levels 100 times lower (Figure 1B).

The relative lack of D-glutamic acid is one of the more intriguing results in this study, particularly since L-glutamic acid is the most prevalent amino acid and all other lower abundance amino acids show appreciable levels of their D-antipodes. D-Glutamic acid is enzymatically converted to D-pyrrolidone carboxylic acid (in rat liver and kidney) and then excreted.⁴⁷ The concurrent high level of D-glutamine may indicate that it is a product of an active D-glutamate reaction or removal pathway. However, unlike the L-glutamate to L-glutamine cycle, this D-glutamate to D-glutamine pathway is largely a unidirectional process.

D-Glutamate and D-aspartate also are metabolized by D-aspartate oxidase (DDO) in mammals.⁴⁸ However, in the present work, D-aspartate levels are high even though DDO is more selective to D-aspartate than to D-glutamate. Therefore, it is likely that the additional processes that involve D-glutamine are limiting D-glutamate levels in the hippocampus and cortex tissues. D-Glutamate is taken up by glial cells and converted via glutamine synthetase to D-glutamine.⁴⁹ Glutamine synthetase is not enantiospecific and can catalyze aminations of both L- and D-glutamate.⁴⁹ However, there are no reports showing that glutaminase, which converts L-glutamine into L-glutamate, is permissive for D-glutamine. This supports the conclusion that the D-glutamate pathway is largely a unidirectional process and not part of a cycle, like the L-glutamate–L-glutamine cycle. The relevance/importance of having low D-glutamate levels is unclear. D-Glutamine is a biomarker for kidney injuries.⁵⁰ In the brain, D-glutamine increased the uptake of tryptophan.⁵¹ D-Glutamine also appeared to produce retrograde amnesia and seizures.⁵² Further work is needed to delineate the impact of errors in endogenous metabolism of D-glutamate.

2.2.2. D-Aspartic Acid (Aspartate) and D-Serine—Levels of D-aspartate and D-serine in the mouse hippocampus and cortex tissues are high compared to all measured D-amino acids, except for D-glutamine (see Figure 1B). The high levels of D-serine and D-aspartate are assumed to be due to their function as neurotransmitters.^{18,20} D-Serine is probably the most studied D-amino acid. Reviews by Wolosker and Schell thoroughly discuss D-serine function in regard to the NMDA receptor.^{53,54} High blood levels of D-aspartate may indicate the need for different tissues to have ready access to this amino acid for a variety of metabolic or signaling processes (and, perhaps, inefficiency of its intracellular synthesis). D-Aspartate has different properties as a neurotransmitter compared

to D-serine.¹⁸ D-Aspartate is present in high concentrations in synaptic vesicles of axon terminals, and the origin of D-aspartate occurs in neurons via D-aspartate racemases.^{18,34} D-Aspartate is involved in synthesis and release of testosterone and luteinizing hormone in rat pituitary gland.^{55,56} In addition, free D-aspartic acid is found in white and gray matter in human brains, but free D-aspartic acid levels are twice as high in gray matter with subjects diagnosed with Alzheimer's disease.^{57,58} More recently, D-aspartate was found to regulate neuronal dendritic morphology, synaptic plasticity, gray matter volume, and brain activity in rats.^{59,60}

2.2.3. D-Branched Chain Amino Acids (D-Leucine, D-Valine, D-Isoleucine, and D-Allo-isoleucine)—The branched chain aliphatic amino acids leucine, isoleucine, allo-isoleucine, and valine all have low total amino acid levels but high % D-amino acid levels (Table 2 and Figure 4). Total levels of branched chain amino acids (BCAAs) are similar in blood compared to those in perfused and nonperfused tissues in most cases, but D-amino acid levels for BCAAs are significantly higher in hippocampus and cortex tissues than blood, as are all the other amino acids in this study (Tables 1–3). No explanation of BCAA levels compared to other amino acids was found in the literature. Recently, it has been discovered that D-leucine, but not D-valine, can be used to treat seizures in mice.²⁵ The levels of D-branched chain amino acids (D-BCAA) in the brain are fairly similar (Figure 1B). In view of D-leucine's use in the treatment of seizures, it may be worth examining the action of other D-BCAAs. Prolinase, which is present in brain tissue, is strongly inhibited by L-BCAAs.⁶¹ Conversely, D-BCAAs enhance prolinase's function.⁶¹ In glial-enriched cultures from mouse brain tissues, D-valine has been used in media to inhibit growth of fibroblasts, causing cultures to be characterized as over 80% astrocytic and having suppressed growth rates.⁶² The results of this experiment suggest that D-valine might have an influence on the growth of select nonglial cell populations in the brain. There are no reports for function of D-allo-isoleucine in mammalian tissues, but levels of L-allo-isoleucine are increased in patients suffering from maple syrup urine disease (the pathophysiological significance of this finding is unclear).^{63,64}

2.2.4. D-Phenylalanine, D-Alanine, and D-Asparagine—The % D-amino acid values of D-asparagine and D-phenylalanine in brain tissues were relatively high (~10–30% range), even though their total amino acid concentrations were comparatively low (Table 2). D-Asparagine and D-phenylalanine exhibited similar concentrations. D-Phenylalanine may alleviate neurological stress through trapping of reactive oxygen species in neurological tissue.⁶⁵ D-Phenylalanine also may treat emotional stress.⁶⁶ Free D-asparagine has not been reported to be involved in any neurological process. However, hydrolysis converts D-asparagine to D-aspartate. D-Aspartate function has been reported in brain tissue (see Section 2.2.2). A D-asparagine to D-aspartate cycle analogous to the glutamate to glutamine cycle has not been identified. D-Alanine showed moderate levels of total amino acid, D-amino acid, and % D-amino acid values in this study. D-Alanine levels were found to be the same in brain white matter of tissue from normal patients and those with Alzheimer's disease, but D-alanine levels were twice as high in Alzheimer's gray matter compared to normal gray matter.⁵⁸ D-Alanine administration can lead to hypofunction of NMDA

neurotransmission, and it has been used as a potential approach for the pharmacotherapy of schizophrenia.⁶⁷

3. CONCLUSIONS

This study provides the most complete baseline analysis of L and D-amino acids in mouse brain tissues to date. Perfusion of brain tissue reduces the variation in brain amino acid levels among mice and suggests that free amino acid data obtained from perfused tissue may give a more accurate measure of cellular and extracellular/extravascular levels. There is little difference in the total amino acid levels in the hippocampus and cortex, but there is an approximate 13% reduction in almost all measured D-amino acid levels in the cortex compared to the hippocampus. An exception is D-aspartic acid, which is 2 times higher in the cortex and has higher blood levels than all other D-amino acids, thereby making it more available to all tissues. Results suggest that additional processes exist other than DAO and racemases that control broad levels of D-amino acids in brain tissues. Plots of % D-amino acids indicate that there is an approximate inverse relationship between the prevalence of an amino acid and the percentage of its D-enantiomeric form. This suggests that there might be other D-amino acid racemases in mammalian brains that underlie the high % D-amino acid values. Total levels of BCAAs are similar in blood compared to hippocampus and cortex tissues, but D-amino acid levels for BCAAs are significantly higher in hippocampus and cortex tissues than blood. A notable result is that glutamic acid, the most prevalent of all free amino acids, had no measurable level of its D-antipode. It is possible that the D-glutamate metabolism is a unidirectional process and not a cycle (i.e., in comparison to the L-glutamate–glutamine cycle). The specific and efficient suppression/removal of D-glutamic acid from the brain is likely to be physiologically important and warrants further investigation.

4. METHODS

4.1. Materials

All amino acid standards, teicoplanin, fluorenylme-thyloxycarbonyl chloride, amantadine hydrochloride, and boric acid were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Sigma-Aldrich, and deionized water was obtained from a Milli-Q water system (Millipore, Bedford, MA). An octadecylsilane derivatized superficially porous particle (SPP)-based HPLC column (Poroshell 120 EC-C18, 4.6 × 150 mm i.d. 2.7 μm particles) was purchased from Agilent Technologies (Wilmington, DE). Another HPLC column was prepared in-house utilizing teicoplanin covalently bonded to SPPs and slurry packed into a 4.6 × 100 mm i.d. stainless steel column (IDEX Health and Science, Oak Harbor, WA).

4.2. Derivatization of Amino Acid Standards

Fluorenylme-thyloxycarbonyl chloride (Fmoc) was used as a fluorescent reagent for amino acid derivatization in this study. Fmoc amino acids have been reported to be stable.⁶⁸ Other fluorescent reagents for precolumn derivatization such as *o*-phthalaldehyde (OPA) and 7-

fluoro-4-nitrobenzo-2-oxa-1,3-diazol (NBD-F) have been reported to form unstable amino acid adducts.⁶⁸ The derivatization of standards was performed in autosampler vials. Standard L- and D-amino acids were prepared in deionized water at concentrations around 0.03 M. Into the autosampler vial, 50 μL of amino acid standards was pipetted. A 0.8 M borate buffer was prepared with boric acid and potassium chloride. The pH of the borate buffer was adjusted with 0.8 M NaOH to pH 9. Into the autosampler vial, 400 μL of borate buffer was pipetted. In addition, 500 μL of acetonitrile was pipetted into the autosampler vial. A Fmoc solution was prepared by dissolving 0.13 g in 5 mL of acetonitrile (0.1 M), and 50 μL of the Fmoc solution was pipetted into the autosampler vial. The mixture was then allowed to react at room temperature after the addition of the Fmoc solution for 20 min. After the reaction was completed, 50 μL of 0.8 M amantadine solution was added to the autosampler vial to quench the remaining Fmoc reagent. The 0.8 M amantadine solution was prepared with acetonitrile and water as a 1:1 mixture. All standards, reagents, and solutions were stored at 4 °C while not in use.

4.3. Mouse Brain Nonperfused Tissues

The study was carried out under experimental protocols approved by the Johns Hopkins Animal Care and Use Committee (ACUC). Reporting of this work complies with ARRIVE guidelines. All efforts were made to minimize animal suffering. In all experiments, NIH Swiss mice (NCI, Frederick, MD) aged 5–6 weeks (body weight 25–30 g) were used. The mice were housed 3–5 per cage, with a simulated 14 h light/10 h dark cycle. The mice were fed a rodent chow diet (Teklad Global 2018SX, Madison, WI) and tap water *ad libitum*.

Mice were sacrificed by rapid cervical dislocation. Surgical scissors were used to remove the head and to complete the brain dissection. The hippocampus and cortex were dissected using a dissecting microscope. All samples were stored at –80 °C until analysis.

4.4. Mouse Brain Perfused Tissues

Mice were anesthetized lightly with carbon dioxide. A midline incision was made at the thoracic costal margin, followed by visualization and incision of the right atrium. Heparin/saline (APP Pharmaceuticals, LLC 1000 USP units/mL, Schaumburg, IL) was injected using a 25-gauge butterfly needle (BD Vacutainer, Four Oaks, NC) into the apex of the left ventricle until a swelling of the heart was observed. The injection was thereafter continued at a low rate. The proximal end of the collection set was removed from the flush syringe when the effluent was clear. A 20 mL syringe was used to slowly inject a 10% neutral buffered formalin (NBF) solution (Sigma Life Science, St. Louis, MO), and when cardiac muscle contraction stopped, perfusion was complete. A 3 mL syringe and 25-gauge one-inch needle were used to infuse the intestines and lungs with 10% NBF, working from the proximal to distal end. Brains were then dissected rapidly according to the procedure previously described. All samples were stored at –80 °C until analysis.

4.5. Blood Samples

Mice were anesthetized with carbon dioxide, and blood was collected rapidly by a cardiac puncture technique using a 22-gauge needle. The blood was collected and stored at –80 °C until analysis.

4.6. Animal Subjects

A total of 19 male mice (NIH Swiss) were utilized for the analysis of 12 free amino acids in hippocampus tissue, cortex tissue, and blood samples. This strain of mice was used in parallel with other experiments on the efficacy of D-leucine in treating epilepsy. Only male mice were analyzed for L- and D-amino acids because the parallel experiments on the efficacy of D-leucine in treating epilepsy showed some adverse effects in female mice. Seven mice were utilized for the nonperfused tissue analysis of the hippocampus and cortex. Another seven mice were utilized for the perfused tissue analysis of the hippocampus and cortex. The last five mice were used for blood analysis (blood was not collected from mice sacrificed for brain tissue because of the need for very rapid tissue/blood acquisition in order to minimize D-amino acid degradation). A total of 14 samples were used for tissue analysis from both perfused and nonperfused mice (seven hippocampus tissue samples and seven cortex tissue samples).

4.7. Free Amino Acid Extraction

The hippocampus, cortex, and blood samples obtained from the dissection and collection processes were weighed and placed into microcentrifugation tubes. To all samples was added 100 μL of an internal standard. The internal standard consisted of 8.38 mM racemic norleucine in water. Next, 1 mL of 0.1 N perchloric acid was pipetted into the tube. Then the samples were homogenized for 30 s (three 10 s pulses) with a Q-Sonica CL-18 probe (Newtown, CT). The samples were placed on ice during the homogenization process. After homogenization, the samples were centrifuged at 13 000 rpm for 20 min at 4 °C. The supernatant was removed and stored at -80°C while not in use. Aliquots (100 μL) of supernatant were derivatized by following the derivatization procedure described for the amino acid standards.

4.8. Two-Dimension HPLC Instrumentation and Method

The chromatography system consisted of an Agilent 1200 HPLC system (Santa Clara, CA) and an LC system consisting of a Shimadzu LC-6A pump, RF-10A fluorescence detector, and CR-6A integrator (Kyoto, Japan). A Rheodyne 7000 six-port stream switching valve (Rohnert Park, CA) was used for the heart-cut from the first dimension to the second dimension. The first dimension utilized a C18 SPP column, and the second dimension utilized an in-house constructed chiral teicoplanin SPP column. Both columns were described in Section 4.1. First dimension signal monitoring was done using ChemStation software from Agilent, and the second-dimension signal was monitored by a CR-6A integrator from Shimadzu. Two reverse-phase HPLC gradients for individual amino acid isolation was performed in the first dimension. The first gradient consisted of mobile phase A (20 mM H_2PO_4 buffer adjusted to pH 2.5 with H_3PO_4) and mobile phase B (acetonitrile). The gradient method began with 5% B (0–2 min) followed by a linear ramp from 15 to 80% B (2.01–35 min) and then 80–95% B (35–38 min). Finally, the gradient concluded with a 2 min ramp down to 5% B. The flow rate was 0.75 mL/min. For the second reverse-phase gradient, mobile phase A was 0.025 M sodium acetate and mobile phase B was a 23/22 (v/v) mixture of 0.05 M sodium acetate/acetonitrile. The gradient method began with a ramp from 30 to 37% B (0–3.75 min) followed by a ramp from 37 to 73% B (3.75–26.25 min) and was

finally brought to 100% B over the concluding 5 min. For the first dimension, a diode array detector (DAD) monitored signals at 254 nm and the detector outlet was connected to the six-port switching valve. Effluent bands were manually cut or redirected to the second dimension column. Manual cuts lasted approximately 0.1 to 1 s. In the second dimension, amino acid enantiomers were separated on the teicoplanin SPP column using isocratic reverse-phase methods. Table S1 lists the conditions for the separation of each chiral amino acid in the second dimension (see the Supporting Information). For the second dimension, fluorometric detection of FMOC-amino acids was conducted using excitation wavelength of 254 nm and an emission wavelength of 313 nm. Figure S1 shows typical results obtained from the chromatographic separations from the first and second dimensions (see the Supporting Information).

The method described was able to evaluate 16 amino acids (Table S1 and Figure S1). The levels of 12 free amino acids were reported in the blood and brain tissues, and representative chromatograms are shown in the Supporting Information (Figure S2). Four of the amino acid levels were below the detection limits (LODs) of our method and therefore could not be reported. Method detection validation of L- and D-amino acids in the samples was determined via mass spectrometry and diode array UV peak purity assay (Figures S3 and S4). Other methods also reported low detection levels for similar amino acids in rat brains (e.g., cysteine, tyrosine, methionine, and D-glutamate).^{69,48}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported by the Robert A. Welch Foundation (Y0026; D.W.A.) and the National Institute of Neurological Disorders and Stroke (K08NS070931; A.L.H.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the Department of Health and Human Services, the United States Government, National Institute of Neurological Disorders and Stroke, or the National Institutes of Health.

We thank AZYP, LLC, for their technical support for HPLC chiral column technology. We would also like to thank the Shimadzu Center for Advanced Analytical Chemistry for the use of a fluorescence detector and Agilent Technologies for providing HPLC superficially porous particle columns.

ABBREVIATIONS

HPLC	high-performance liquid chromatography
DAD	diode array detector
NMDA	<i>N</i> -methyl-D-aspartate
DAO	D-amino acid oxidase
ddy/DAO–	mice lacking D-amino acid oxidase
ddy/DAO+	wild-type mice

CBV	cerebral blood volume
DOD	D-aspartate oxidase
BCAAs	branched chain amino acids
D-BCAAs	D-branched chain amino acids
SPP	superficially porous particle
Fmoc	fluorenylmethyloxycarbonyl chloride
NBF	neutral buffered formalin
OPA	o-phthaldialdehyde
NBD-F	7-fluoro-4-nitrobenzo-2-oxa-1,3-diazol

References

1. Vauquelin LN, Robiquet PJ. The discovery of a new plant principle in *Asparagus sativus*. *Ann Chim.* 1806; 57:88–93.
2. Vickery HB, Schmidt CLA. History of the discovery of the amino acids. *Chem Rev.* 1931; 9:169–318.
3. Gal J. Louis Pasteur, language, and molecular chirality. I Background and Dissymmetry. *Chirality.* 2011; 23:1–16. [PubMed: 20589938]
4. Greenstein JP, Birnbaum SM, Otey MC. Optical and enzymatic characterization of amino acids. *J Biol Chem.* 1953; 204:307–21. [PubMed: 13084603]
5. Corrigan JJ. D-Amino acids in animals. *Science.* 1969; 164:142–9. [PubMed: 5774186]
6. Cline DB. On the determination of the physical origin of homochirality in life. *Comments Nucl Part Phys.* 1997; 22:131–154.
7. Stevens CM, Halpern PE, Gigger RP. Occurrence of D-amino acids in some natural materials. *J Biol Chem.* 1951; 190:705–10. [PubMed: 14841221]
8. Armstrong DW, Gasper M, Lee SH, Zukowski J, Ercal N. D-Amino acid levels in human physiological fluids. *Chirality.* 1993; 5:375–8. [PubMed: 8398594]
9. Armstrong DW, Duncan JD, Lee SH. Evaluation of D-amino acid levels in human urine and in commercial L-amino acid samples. *Amino Acids.* 1991; 1:97–106. [PubMed: 24194052]
10. Aswad DW. Determination of D- and L-aspartate in amino acid mixtures by high-performance liquid chromatography after derivatization with a chiral adduct of o-phthaldialdehyde. *Anal Biochem.* 1984; 137:405–409. [PubMed: 6731824]
11. D'Aniello A. D-Aspartic acid: an endogenous amino acid with an important neuroendocrine role. *Brain Res Rev.* 2007; 53:215–234. [PubMed: 17118457]
12. Dunlop DS, Neidle A, McHale D, Dunlop DM, Lajtha A. The presence of free D-aspartic acid in rodents and man. *Biochem Biophys Res Commun.* 1986; 141:27–32. [PubMed: 3801000]
13. Armstrong DW, Zukowski J, Ercal N, Gasper M. Stereochemistry of pipecolic acid found in the urine and plasma of subjects with peroxisomal deficiencies. *J Pharm Biomed Anal.* 1993; 11:881–6. [PubMed: 8305590]
14. Janecka A, Fichna J, Janecki T. Opioid receptors and their ligands. *Curr Top Med Chem.* 2004; 4:1–17. [PubMed: 14754373]
15. Heck SD, Siok CJ, Krapcho KJ, Kelbaugh PR, Thadeio PF, Welch MJ, Williams RD, Ganong AH, Kelly ME, et al. Functional consequences of posttranslational isomerization of Ser46 in a calcium channel toxin. *Science.* 1994; 266:1065–8. [PubMed: 7973665]
16. Bathena SP, Huang J, Epstein AA, Gendelman HE, Boska MD, Alnouti Y. Rapid and reliable quantitation of amino acids and myo-inositol in mouse brain by high performance liquid

- chromatography and tandem mass spectrometry. *J Chromatogr B: Anal Technol Biomed Life Sci.* 2012; 893–894:15–20.
17. Lu X, Lu J, Liu CW, Zhao SL. Determination of D-aspartic acid and D-glutamic acid in midbrain of Parkinson's disease mouse by reversed phase high performance liquid chromatography. *Fenxi Huaxue.* 2007; 35:1151–1154.
18. D'Aniello S, Somorjai I, Garcia-Fernandez J, Topo E, D'Aniello A. D-Aspartic acid is a novel endogenous neurotransmitter. *FASEB J.* 2011; 25:1014–1027. [PubMed: 21163862]
19. Fuchs SA, Berger R, Klomp LWJ, de Koning TJ. D-amino acids in the central nervous system in health and disease. *Mol Genet Metab.* 2005; 85:168–180. [PubMed: 15979028]
20. Mothet JP, Parent AT, Wolosker H, Brady RO Jr, Linden DJ, Ferris CD, Rogawski MA, Snyder SH. D-Serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. *Proc Natl Acad Sci U S A.* 2000; 97:4926–4931. [PubMed: 10781100]
21. Hashimoto A, Nishikawa T, Hayashi T, Fujii N, Harada K, Oka T, Takahashi K. The presence of free D-serine in rat brain. *FEBS Lett.* 1992; 296:33–6. [PubMed: 1730289]
22. Nagata Y, Horiike K, Maeda T. Distribution of free D-serine in vertebrate brains. *Brain Res.* 1994; 634:291–5. [PubMed: 8131079]
23. Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T, Takashima S, Doi N, Mizutani Y, et al. Embryonic development and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex. *J Neurochem.* 1993; 61:348–51. [PubMed: 8515283]
24. Moaddel R, Luckenbaugh DA, Xie Y, Villaseñor A, Brutsche NE, Machado-Vieira R, Ramamoorthy A, Lorenzo MP, Garcia A, Bernier M, Torjman MC, Barbas C, Zarate CA, Wainer IW. D-serine plasma concentration is a potential biomarker of (R,S)-ketamine antidepressant response in subjects with treatment-resistant depression. *Psychopharmacology.* 2015; 232:399–409. [PubMed: 25056852]
25. Hartman AL, Santos P, O'Riordan KJ, Stafstrom CE, Marie Hardwick J. Potent anti-seizure effects of D-leucine. *Neurobiol Dis.* 2015; 82:46–53. [PubMed: 26054437]
26. Hamase K, Morikawa A, Ohgusu T, Lindner W, Zaitzu K. Comprehensive analysis of branched aliphatic D-amino acids in mammals using an integrated multi-loop two-dimensional column-switching high-performance liquid chromatographic system combining reversed-phase and enantioselective columns. *Journal of Chromatography A.* 2007; 1143:105–111. [PubMed: 17223114]
27. Hamase K, Miyoshi Y, Ueno K, Han H, Hirano J, Morikawa A, Mita M, Kaneko T, Lindner W, Zaitzu K. Simultaneous determination of hydrophilic amino acid enantiomers in mammalian tissues and physiological fluids applying a fully automated micro-two-dimensional high-performance liquid chromatographic concept. *Journal of Chromatography A.* 2010; 1217:1056–1062. [PubMed: 19767006]
28. Gage GJ, Kipke DR, Shain W. Whole Animal Perfusion Fixation for Rodents. *J Visualized Exp.* 2012:3564.
29. Sasabe J, Suzuki M, Imanishi N, Aiso S. Activity of D-amino acid oxidase is widespread in the human central nervous system. *Front Synaptic Neurosci.* 2014; 6:14. [PubMed: 24959138]
30. Chugh BP, Lerch JP, Yu LX, Pienkowski M, Harrison RV, Henkelman RM, Sled JG. Measurement of cerebral blood volume in mouse brain regions using micro-computed tomography. *NeuroImage.* 2009; 47:1312–8. [PubMed: 19362597]
31. Friedman, M. D-Amino Acids in Chemistry, Life Sciences, and Biotechnology. Verlag Helvetica Chimica Acta; 2011. Origin, microbiology, nutrition, and pharmacology of D-amino acids; p. 173–212.
32. Armstrong DW, Gasper MP, Lee SH, Ercal N, Zukowski J. Factors controlling the level and determination of D-amino acids in the urine and plasma of laboratory rodents. *Amino Acids.* 1993; 5:299–315. [PubMed: 24190673]
33. Wolosker H, Blackshaw S, Snyder SH. Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. *Proc Natl Acad Sci U S A.* 1999; 96:13409–13414. [PubMed: 10557334]

34. Kim PM, Duan X, Huang AS, Liu CY, Ming GL, Song H, Snyder SH. Aspartate racemase, generating neuronal D-aspartate, regulates adult neurogenesis. *Proc Natl Acad Sci U S A*. 2010; 107:3175–3179. [PubMed: 20133766]
35. Pollegioni L, Piubelli L, Sacchi S, Pilone MS, Molla G. Physiological functions of D-amino acid oxidases: from yeast to humans. *Cell Mol Life Sci*. 2007; 64:1373–1394. [PubMed: 17396222]
36. Moreno S, Nardacci R, Cimini A, Cer MP. Immunocytochemical localization of D-amino acid oxidase in rat brain. *J Neurocytol*. 1999; 28:169–185. [PubMed: 10617900]
37. Bendikov I, Nadri C, Amar S, Panizzutti R, Demiranda J, Wolosker H, Agam G. A CSF and postmortem brain study of D-serine metabolic parameters in schizophrenia. *Schizophr Res*. 2007; 90:41–51. [PubMed: 17156977]
38. Ono K, Shishido Y, Park HK, Kawazoe T, Iwana S, Chung SP, Abou El-Magd RM, Yorita K, Okano M, Watanabe T, Sano N, Bando Y, Arima K, Sakai T, Fukui K. Potential pathophysiological role of D-amino acid oxidase in schizophrenia: immunohistochemical and in situ hybridization study of the expression in human and rat brain. *J Neural Transm*. 2009; 116:1335–1347. [PubMed: 19685198]
39. Horiike K, Tojo H, Arai R, Nozaki M, Maeda T. D-Amino-acid oxidase is confined to the lower brain stem and cerebellum in rat brain: regional differentiation of astrocytes. *Brain Res*. 1994; 652:297–303. [PubMed: 7953743]
40. Schell MJ, Cooper OB, Snyder SH. D-aspartate localizations imply neuronal and neuroendocrine roles. *Proc Natl Acad Sci U S A*. 1997; 94:2013–2018. [PubMed: 9050896]
41. Zaar K, Köst HP, Schad A, Völkl A, Baumgart E, Fahimi HD. Cellular and subcellular distribution of D-aspartate oxidase in human and rat brain. *J Comp Neurol*. 2002; 450:272–282. [PubMed: 12209855]
42. Dixon M, Kleppe K. D-Amino acid oxidase. II Specificity, competitive inhibition, and reaction sequence. *Biochim Biophys Acta, Nucleic Acids Protein Synth*. 1965; 96:368–82.
43. Madeira C, Freitas ME, Vargas-Lopes C, Wolosker H, Panizzutti R. Increased brain D-amino acid oxidase (DAAO) activity in schizophrenia. *Schizophrenia Research*. 2008; 101:76–83. [PubMed: 18378121]
44. Meldrum BS. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J Nutr*. 2000; 130:1007S–1015S. [PubMed: 10736372]
45. Daikhin Y, Yudkoff M. Compartmentation of brain glutamate metabolism in neurons and glia. *J Nutr*. 2000; 130:1026S–1031S. [PubMed: 10736375]
46. Shen J, Petersen KF, Behar KL, Brown P, Nixon TW, Mason GF, Petroff OAC, Shulman GI, Shulman RG, Rothman DL. Determination of the rate of the glutamate/glutamine cycle in the human brain by in vivo ¹³C NMR. *Proc Natl Acad Sci U S A*. 1999; 96:8235–8240. [PubMed: 10393978]
47. Wilson WE, Koeppe RE. Metabolism of D- and L-glutamic acid in the rat. *J Biol Chem*. 1961; 236:365–369. [PubMed: 13785721]
48. Han H, Miyoshi Y, Koga R, Mita M, Konno R, Hamase K. Changes in D-aspartic acid and D-glutamic acid levels in the tissues and physiological fluids of mice with various D-aspartate oxidase activities. *J Pharm Biomed Anal*. 2015; 116:47–52. [PubMed: 26058797]
49. Pow DV, Crook DK. Direct immunocytochemical evidence for the transfer of glutamine from glial cells to neurons: use of specific antibodies directed against the D-stereoisomers of glutamate and glutamine. *Neuroscience (Amsterdam, Neth)*. 1996; 70:295–302.
50. Liang Q, Liu H, Xing H, Jiang Y, Zhang T, Zhang AH. High-resolution mass spectrometry for exploring metabolic signatures of sepsis-induced acute kidney injury. *RSC Adv*. 2016; 6:29863–29868.
51. Gorgievski-Hrisoho M, Colombo JP, Bachmann C. Stimulation of tryptophan uptake into brain microvessels by D-glutamine. *Brain Res*. 1986; 367:395–7. [PubMed: 3697715]
52. Davis JL, Cherkin A. D-Glutamine produces seizures and retrograde amnesia in the chick. *Pharmacol, Biochem Behav*. 1981; 15:367–369. [PubMed: 7197365]
53. Wolosker H. NMDA receptor regulation by D-serine: new findings and perspectives. *Mol Neurobiol*. 2007; 36:152–164. [PubMed: 17952659]

54. Schell MJ. The N-methyl D-aspartate receptor glycine site and D-serine metabolism: an evolutionary perspective. *Philos Trans R Soc, B*. 2004; 359:943–964.
55. D’Aniello A, Di Fiore MM, Fisher GH, Milone A, Seleni A, D’Aniello S, Perna AF, Ingrosso D. Occurrence of D-aspartic acid and N-methyl-D-aspartic acid in rat neuroendocrine tissues and their role in the modulation of luteinizing hormone and growth hormone release. *FASEB J*. 2000; 14:699–714. [PubMed: 10744627]
56. D’Aniello A, Cosmo AD, Cristo CD, Annunziato L, Petrucelli L, Fisher G. Involvement of D-Aspartic acid in the synthesis of testosterone in rat testes. *Life Sci*. 1996; 59:97–104. [PubMed: 8699926]
57. D’Aniello A, Lee JM, Petrucelli L, Di Fiore MM. Regional decreases of free D-aspartate levels in Alzheimer’s disease. *Neurosci Lett*. 1998; 250:131–134. [PubMed: 9697936]
58. Fisher GH, D’Aniello A, Vetere A, Padula L, Cusano GP, Man EH. Free D-aspartate and D-alanine in normal and Alzheimer brain. *Brain Res Bull*. 1991; 26:983–5. [PubMed: 1933416]
59. Topo E, Soricelli A, Di Maio A, D’Aniello E, Di Fiore MM, D’Aniello A. Evidence for the involvement of D-aspartic acid in learning and memory of rat. *Amino Acids*. 2010; 38:1561–1569. [PubMed: 19890700]
60. Errico F, Nistico R, Di Giorgio A, Squillace M, Vitucci D, Galbusera A, Piccinin S, Mango D, Fazio L, Middei S, Trizio S, Mercuri NB, Teule MA, Centonze D, Gozzi A, Blasi G, Bertolino A, Usiello A. Free D-aspartate regulates neuronal dendritic morphology, synaptic plasticity, gray matter volume and brain activity in mammals. *Transl Psychiatry*. 2014; 4:e417. [PubMed: 25072322]
61. Wang W, Liu G, Yamashita K, Manabe M, Kodama H. Characteristics of prolinase against various iminodipeptides in erythrocyte lysates from a normal human and a patient with prolidase deficiency. *Clin Chem Lab Med*. 2004; 42:1102–1108. [PubMed: 15552267]
62. Estin C, Vernadakis A. Primary glial cells and brain fibroblasts: Interactions in culture. *Brain Res Bull*. 1986; 16:723–731. [PubMed: 2874876]
63. Matthews DE, Ben-Galim E, Haymond MW, Bier DM. Alloisoleucine formation in maple syrup urine disease: isotopic evidence for the mechanism. *Pediatr Res*. 1980; 14:854–857. [PubMed: 7413299]
64. Wendel U, Langenbeck U, Seakins JW. Interrelation between the metabolism of L-isoleucine and L-allo-isoleucine in patients with maple syrup urine disease. *Pediatr Res*. 1989; 25:11–4. [PubMed: 2919111]
65. Oeckl P, Ferger B. Analysis of hydroxylation and nitration products of D-phenylalanine for in vitro and in vivo radical determination using high-performance liquid chromatography and photodiode array detection. *J Chromatogr B: Anal Technol Biomed Life Sci*. 2009; 877:1501–1508.
66. Yumatov AE, Sarychev EI, Kozlovskii II, Mineeva MF, Demidov VM, Morozov IS, Kozlovskaya MM. Increasing stability to emotional stress by means of D-phenylalanine. *Zh Vyssh Nervn Deyat im I P Pavlova*. 1991; 41:156–161.
67. Tsai GE, Yang P, Chang YC, Chong MY. D-alanine added to antipsychotics for the treatment of schizophrenia. *Biol Psychiatry*. 2006; 59:230–234. [PubMed: 16154544]
68. Masuda A, Dohmae N. Amino acid analysis of sub-picomolar amounts of proteins by precolumn fluorescence derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. *BioSci Trends*. 2011; 5:231–238. [PubMed: 22281536]
69. Mangas A, Coveñas R, Bodet D, Geffard M, Aguilar LA, Yajeya J. Immunocytochemical visualization of d-glutamate in the rat brain. *Neuroscience*. 2007; 144:654–664. [PubMed: 17084987]

Figure 1.

(A) Average values of total amino acid levels ($\mu\text{g}/\text{mg}$) in blood, nonperfused cortex, and nonperfused hippocampus. The range of the average values can be seen in Table 2. Average value for blood with $n = 5$. Average value for nonperfused cortex with a $n = 7$. Average value for nonperfused hippocampus with $n = 7$. (B) Average values of D-amino acid levels ($\mu\text{g}/\text{mg}$) in blood, nonperfused cortex, and nonperfused hippocampus. The range of the average values can be seen in Table 2. Average value for blood with $n = 5$. Average value for nonperfused cortex with a $n = 7$. Average value for nonperfused hippocampus with $n = 7$.

Figure 2.

Linear plots of the total amino acid levels in perfused and nonperfused samples and normalized data (with respect to glutamic acid) in comparison to raw data. (A) Hippocampus plot of normalized total amino acid levels of perfused tissue vs nonperfused tissue. (B) Hippocampus plot of raw total amino acid levels of perfused tissue vs nonperfused tissue. (C) Cortex plot of normalized total amino acid levels of perfused tissue vs nonperfused tissue. (D) Cortex plot of raw total amino acid levels of perfused tissue vs nonperfused tissue.

Figure 3.

(A) Comparison of total amino acid levels in the cortex vs hippocampus. (B) Comparison of D-amino acid levels in the cortex vs hippocampus; Asp, circled in red, is not included in the calibration. In both plots, each data point represents an individual amino acid value that is averaged from $N=7$.

Figure 4. (A) Plot of hippocampus % D amino acid levels vs total amino acid levels. (B) Plot of cortex % D amino acid levels vs total amino acid levels. In both plots, each data point represents an individual amino acid value that is averaged from $N=7$.

Table 1
Total Amino Acid and D-Amino Acid Levels in Nonperfused Cortex and Hippocampus^a

	cortex				hippocampus					
	total amino acid (μg/mg)		D-amino acid (μg/mg)		total amino acid (μg/mg)		D-amino acid (μg/mg)			
	range	average	range	average	range	average	range	average		
Leu	0.02–0.05	0.04	0.0005–0.007	0.004	12.1	0.03–0.14	0.08	0.002–0.053	0.013	16.3
Ser	0.11–0.49	0.25	0.034–0.139	0.076	30.9	0.11–0.6	0.28	0.04–0.163	0.095	34.2
Ala	0.18–0.34	0.25	0.0003–0.019	0.010	3.9	0.17–0.51	0.32	0.003–0.079	0.031	9.7
Asp	0.46–1.82	0.94	0.003–0.283	0.115	12.2	0.36–1.58	0.77	0.005–0.042	0.036	4.6
Thr	0.04–0.20	0.10	0.0023–0.022	0.007	7.6	0.05–0.31	0.14	0.003–0.050	0.017	12.1
Glu	1.57–8.10	3.90	<0.1 to <0.1	<0.1	<0.1	1.37–8.69	3.50	<0.1 to <0.1	<0.1	<0.1
Val	0.02–0.12	0.05	0.0003–0.027	0.011	21.0	0.04–0.22	0.11	0.005–0.061	0.023	21.3
Asn	0.02–0.09	0.04	0.0004–0.011	0.004	10.5	0.02–0.33	0.11	0.001–0.091	0.014	12.2
Gln	0.81–2.5	1.48	0.001–0.330	0.117	7.9	0.67–3.09	1.43	0.00002–0.032	0.012	0.8
Ile	0.001–0.03	0.01	0.0002–0.005	0.003	26.1	0.002–0.09	0.03	0.0004–0.027	0.007	22.9
Allo-Ile	0.0004–0.005	0.003	0.0002–0.0007	0.001	16.9	0.0002–0.032	0.01	0.0001–0.005	0.004	47.0
Phe	0.02–0.05	0.03	0.0003–0.019	0.005	15.1	0.01–0.3	0.09	0.0007–0.050	0.021	22.8

^a 14 samples were analyzed from 7 mice.

^b Average D % = D/(D + L) × 100%.

Table 2
Total Amino Acid and D-Amino Acid Levels in Perfused Cortex and Hippocampus^a

	cortex				hippocampus			
	total amino acid (μg/mg)		D-amino acid (μg/mg)		total amino acid (μg/mg)		D-amino acid (μg/mg)	
	range	average	range	average	range	average	range	average
Leu	0.02–0.05	0.04	0.002–0.009	0.006	0.02–0.08	0.04	0.002–0.030	0.009
Ser	0.08–0.25	0.15	0.024–0.073	0.047	0.09–0.28	0.17	0.029–0.078	0.053
Ala	0.16–0.38	0.25	0.006–0.053	0.025	0.17–0.37	0.26	0.003–0.063	0.034
Asp	0.41–1.40	0.79	0.039–0.080	0.106	0.34–1.19	0.65	0.030–0.097	0.064
Thr	0.02–0.10	0.05	0.002–0.007	0.006	0.04–0.17	0.08	0.001–0.018	0.007
Glu	2.08–5.02	3.34	<0.1 to <0.1	<0.1	1.71–4.40	2.96	<0.1 to <0.1	<0.1
Val	0.02–0.03	0.02	0.001–0.005	0.003	0.02–0.05	0.05	0.005–0.011	0.009
Asn	0.01–0.05	0.03	0.001–0.014	0.007	0.03–0.11	0.05	0.001–0.053	0.014
Gln	0.65–1.86	1.19	0.001–0.149	0.048	0.62–1.73	1.11	0.001–0.123	0.058
Ile	0.001–0.02	0.01	0.00003–0.004	0.001	0.003–0.016	0.01	0.0004–0.006	0.002
Allo-Ile	0.001–0.004	0.002	0.00004–0.0009	0.0005	0.0002–0.004	0.002	0.0001–0.001	0.001
Phe	0.01–0.05	0.03	0.001–0.015	0.005	0.02–0.04	0.03	0.003–0.013	0.008

^a 14 samples were analyzed from 7 mice.

^b Average D % = D/(D + L) × 100%.

Table 3Total Amino Acid and D-Amino Acid Levels in Blood^a

	total amino acid (μ g/mg)		D-amino acid (μ g/mg)		D % ^b	
	range	average	range	average	range	average
Leu	0.034–0.039	0.04	0.00005–0.0001	0.0001	0.1–0.4	0.3
Ser	0.010–0.020	0.01	0.001–0.001	0.001	4.1–7.7	5.8
Ala	0.033–0.065	0.05	0.000005–0.00009	0.00005	0.008–0.2	0.09
Asp	0.157–0.29	0.19	0.03–0.04	0.04	15.3–26.2	18.9
Thr	0.083–0.104	0.10	0.0002–0.003	0.002	0.2–4.0	2.0
Glu	0.17–0.22	0.19	<0.00001 to <0.00001	<0.00001	<0.01 to <0.01	<0.01
Val	0.025–0.032	0.03	0.00007–0.0009	0.0003	0.3–3.6	1.1
Asn	0.009–0.018	0.01	0.0003–0.0006	0.0004	2.8–4.9	3.6
Gln	0.104–0.125	0.12	0.00001–0.0001	0.00005	0.004–0.1	0.04
Ile	0.01–0.012	0.01	0.00002–0.0003	0.0001	0.1–2.8	0.8
allo-Ile	0.0002–0.001	0.0004	0.00001–0.0005	0.0001	2.1–60.0	26.1
Phe	0.014–0.019	0.02	0.001–0.006	0.003	2.9–34.1	15.6

^a 5 blood samples were analyzed from 5 mice.^b Average D % = $D/(D + L) \times 100$.