DETERMINATION OF TOTAL AND D-AMINO ACID CONTENT IN MICE BRAIN TISSUE BY ACHIRAL-CHIRAL HEART-CUTTING TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY: DIAGNOSTIC AND CLINICAL RELEVANCE AND A COMPARISON BETWEEN NON-PERFUSED AND PERFUSED TISSUE

by

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Abstract

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A heart-cutting two dimensional liquid chromatographic method for the quantification of free amino acid enantiomers from mouse brain tissue is demonstrated. Evidence to support the occurrence of D-amino acids in significant concentrations in physiological samples has spurred studies toward the elucidation of their importance and function. In this work, the level and regional distribution of select amino acids were determined by achiral-chiral LC/LC. Additionally, the effect of tissue perfusion on these levels was investigated. The free amino acid extracts were analyzed as their 9-fluorenylmethyloxycarbonyl derivatives and separated in the reverse phase mode on superficially porous C18. In the second dimension, enantiomers of selected amino acids were separated using a house prepared column featuring superficially porous silica derivatized with teicoplanin, a chiral selector known to exhibit excellent selectivity toward isomers of native and N-blocked amino acids. Total concentrations of free amino acids were determined through the use of an internal standard and enantiomeric ratios for 11 standard amino acids were determined with high sensitivity using fluorometric detection.

Because some D-amino acids have been suggested to play a role in neurodevelopment and neurotransmission, and may have potential as disease biomarkers, the interest in their occurrence and disposition has recently grown.

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Chapter 1

AMINO ACIDS: EMERGENCE AND RELEVANCY

1.1 Introduction to Amino Acids

1.1.1 Amino acid

Amino acids comprise a class of organic molecules of paramount biological importance. Though there exist many hundreds of compounds that conform to the structural requirements of amino acids, only a select few are biologically relevant in mammals. In fact, triplet codon sequences intrinsically encode merely twenty amino acids universally designated as standard amino acids. Predictably, the common structural elements include an amine ($-NH_2$) and a carboxylic acid (-COOH). The final feature of the amino acid is a characteristic side chain (-R), the moiety ultimately forecasting its function. The most prevalent orientation of these substituents is one where amine, carboxylic acid, and side chain groups are bound to a common carbon, the α-carbon (Figure 1-1). These twenty genetically coded small molecules abound primarily in polymeric form as proteins, macromolecules with an extraordinary scope of responsibility. Nonetheless, there is recent unequivocal evidence that free and non-proteinogenic amino acids, however less abundant, prove no less vital.

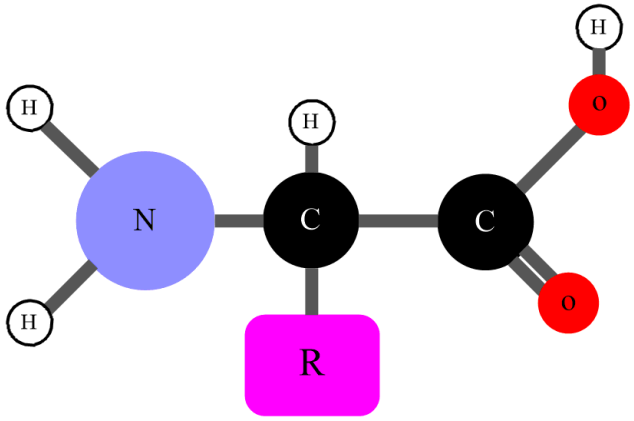

Figure 1-1 General amino acid structure

1.1.2 Optical isomerism and current nomenclature

The history of amino acids began with the aptly named asparagine, isolated from an extract of asparagus in 1806.[1] Over ensuing years, discoveries of this sort amassed to reveal a list of common protein hydrolysates deemed amino acids.[2] Concurrently, Louis Pasteur would demonstrate the unprecedented enantioseparation of (+/-) tartaric acid and establish the correlation between asymmetry and optical activity.[3] His impact extended to amino acid chemistry in 1851 as he revealed the optical activity of naturally occurring asparagine and the related aspartic acid.[4] Accordingly, the common amino acids, with the exception of glycine, occur as optical isomers as a result of their opposing orientation around the α-carbon.[5] The criterion for assignment of relative configuration to natural amino acids is based on the parallelism between the orientation of an asymmetric carbon for the amino acid isomer and that of a known reference. Thus, the L- and D-terminology is one of many contributions to protein chemistry credited to Emil Fischer, who established such a reference when he determined the configurations of asymmetric carbons in various sugars and aldehydes.[4] The accrual of these relevant observations ultimately led to the definitive assertion that naturally occurring amino acids were exclusively of the L-configuration. D-amino acids were simply considered unnatural.

1.2 D-Amino Acids: Early Interest

1.2.1 Paradigm shift

Proteins of living organisms generally incorporate standard L-amino acids almost exclusively. Nonetheless, in the early to mid-20th century, reports emerged describing the isolation of D-amino acids from a variety of antibiotics, including penicillin, and the capsular component of bacterial cells.[6] A representation of this component, peptidoglycan, is characterized by repeating structural units of *N*-acetylglucosamine

(NAG) and *N*-acetylmuramic acid (NAM) cross-linked with D-amino acid containing peptides (Figure 1-2).[7] This polymeric macromolecule is an integral component of the bacterial cell wall to provide structural rigidity, and reports leading to the elucidation of its composition provided the first unambiguous proof that D-amino acids were naturally present and necessary.[8]

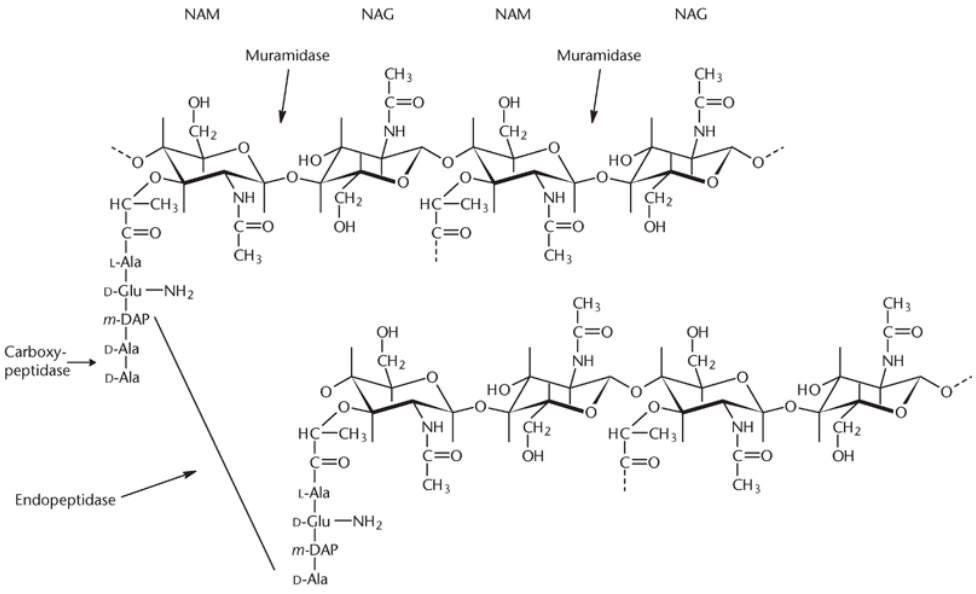

Figure 1-2 Primary structure of peptidoglycan[9]

Evidence then continued to accumulate supporting the natural occurrence of D-amino acids, albeit in a limited and sometimes capricious sense, and by 1969, J. Corrigan published a review describing this accrual of approximately 50 D-amino acids (nonstandard and standard) extant in particular invertebrate species.[10] This work assured the chemical community that biological occurrence of D-amino acids was veritable fact, naturally posing questions regarding their significance. Nevertheless, several years and

numerous analytical innovations proved requisite for a more comprehensive understanding of D-amino acid presence and functionality in mammals.

1.2.2 Occurrence in Nature

Though considerable attention to mammalian production and metabolism of D-amino acids had already been garnered, an explanation regarding their mere presence in nature needed to be addressed. The fundamental concept of nature's asymmetry, specifically with respect to amino acids, had been violated. To this point, D-amino acids were considered laboratory artifacts and deemed unnatural.[10] While it may remain true that life with chiral parity could not exist (e.g. DNA replication would not be possible in a heterochiral system), misguided scientists to this day proclaim that D-amino acids are non-existent in nature.[11] On the contrary, an intrinsic feature of molecules containing a carbonyl group with a stereogenic α-carbon is their ability to undergo base or acid catalyzed racemization (Figure 1-3). This occurs as a cell external process in nature and is dependent on such variables as temperature and pH.[12] Consequently, the "unnatural" variant of amino acids finds itself present in environmental samples, ultimately incorporated into the diet of various organisms.[13]

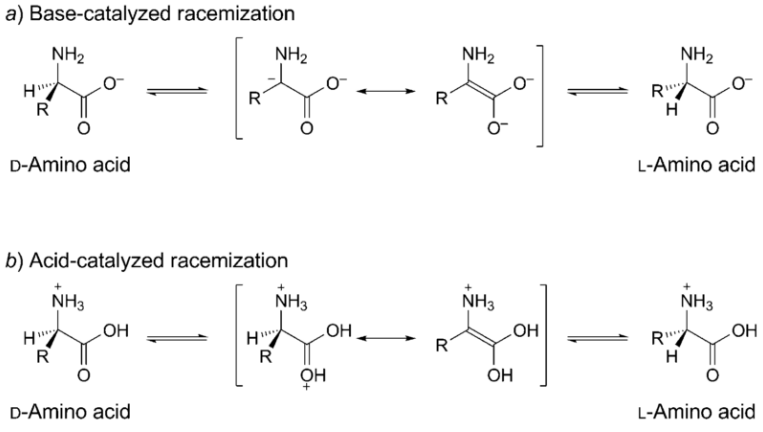

Figure 1-3 Cell external base and acid catalyzed amino acid racemization[12]

Racemization also occurs *in vivo* as evidenced by the occurrence of D-asp residues in metabolically inert protein from various tissues of the elderly.[14] These phenomena outlining the natural occurrence of D-amino acids explains the necessity for the evolution of D-amino acid oxidases and D-amino acid dehydrogenases for the respective eukaryotic and prokaryotic catabolic elimination of D-amino acids. Very recently, D-amino acid racemases have also been identified and credited with the *de novo* production of various free D-amino acids in prokaryotes and eukaryotes.[15] While D-amino acid enzymology is beyond the scope of this discussion, a more extensive description of the physiological importance of free D-amino acids in mammals will be presented in Chapter 2.

1.2.3 Amino Acid Racemization for Geological Dating

When a living organism dies, proteins begin their natural diagenetic breakdown. Peptide bonds hydrolyze and free and proteinogenic amino acids racemize at rates dependent on environmental factors including temperature and pH.[16] Proteins with a greater level of exposure to the environment will typically undergo more rapid and unpredictable diagenesis, whereas intact proteins well preserved in more stable matrices (i.e. carbonate) will undergo the same processes at a slow and predictable rate.[17] The racemization rate of proteinogenic amino acids can thus be derived for a particular environment after calibration with a secondary dating method to determine the appropriate rate constant.[18] In the mid-20th century, this knowledge piqued the interest of chemistry inclined archeologists and spurred the most exciting attention D-amino acids had so far enjoyed. A novel geochronological dating method was born that could accurately date materials too young or too old for the traditionally and more often used radiocarbon method.[19] The amino acid racemization technique and its pioneering scientist, Dr. Jeffrey Bada, would later have their names tarnished after the antiquity of

the Del Mar skull was hugely overestimated due to a faulty calibration and poorly estimated temperature history.[20]

Chapter 2

D-AMINO ACIDS IN MAMMALS

2.1 Advancing Techniques in the Determination of D-amino acids

2.1.1 One dimensional techniques

The sensitive and selective determination of free D-amino acids in biological samples is challenging for several reasons: trace detection of D-amino acids is hindered by high concentrations of L-amino acids, other biologically important amines, peptides, and a variety of small molecules present in such complex matrices. Limitations of the analytical method are therefore magnified due to such inherent difficulties. In the 1980's, D-aspartyl residues were confirmed present in long-lived proteins of the elderly and recently deceased patients where they arise *in situ*.[21,22,23] The results of this work were somewhat fickle due to the harsh conditions with which these proteins were hydrolyzed, undoubtedly inducing racemization to some extent.[24] In 1986, Dunlop et al. applied some of the advancing analytical methods for which the field had waited to determine the concentration of free D-aspartic acid (D-asp) in human and animal tissue.[25] To the day's scientific community, these results were truly unanticipated, for D-amino acids to this point were found only sparingly in tissue of the elderly, and this was the first document confirming free, non-proteinogenic D-amino acid presence in tissue.[25] Methodologies in this work and future investigations were typically performed using a one dimensional high performance liquid chromatography (HPLC) technique via application a chiral derivatizing agent, in this case, (+)-1-(1-naphthyl)ethyl isocyanate. Under alkaline conditions, the isocyanate reacts with the amino acid to yield the corresponding napthyethylcarbamoyl amino acid. Using an enantiomerically pure derivatizing reagent, the separation of the resulting diasteromers can then be favorably achieved using a reverse phase (C18) format. To eliminate ambiguity, Dunlop et al. then applied the (-)-1-(1-naphthyl)ethyl

isocyanate to ensure reversal of the elution order, and the identity of the D-aspartate was somewhat effectively verified. The findings of this work were impactful; high concentrations of D-asp were reported in the young rat cerebral hemisphere (100 nmol/g), kidney, spinal cord, and cerebellum.[25] The regional distribution of free D-asp in tissue of older rats was different from that for young rats, a continual decrease in concentration with increasing age. This was the first implication that free D-asp may have a definitive role in mammalian developmental processes. Six years later, in 1992, Hashimoto et al. reported high concentrations of free D-serine (D-ser) in rat brain tissue using a similar method; to the free amino acid extract was applied pentafluoropropionic anhydride and isopropanol to yield the corresponding N,O-pentafluoropropionylisopropyl amino acid diasteromer. The free D-ser was separated using a gas chromatographic method where problems were encountered due to co-eluting impurities. GC-MS data did however support the occurrence of free D-ser.[26] Acknowledging the shortcomings of this method, the same group applied an HPLC method, again using chiral derivatizing agents; perhaps the most commonly used fluorescent reagent, *o*-phthalaldehyde (OPA) in conjunction with a chiral thiol, N-tert-butyloxycarbonyl-L-cysteine (Boc-L-Cys), were applied and the resulting diasteromers were separated on an octadecylsilyl (ODS) reverse phase column.[27] In these studies, approximately 30% of the total free serine was found to be of the D-configuration. After the discovery of large concentrations of D-asp and D-ser, naturally occurring free D-amino acids truly garnered increased attention from the scientific community. The popular approach was still to apply a chiral derivatizing agent (e.g. OPA in conjunction with chiral thiol; Figure 2-1), the impetus for this being the stark efficiency gains over an enantioselective column.

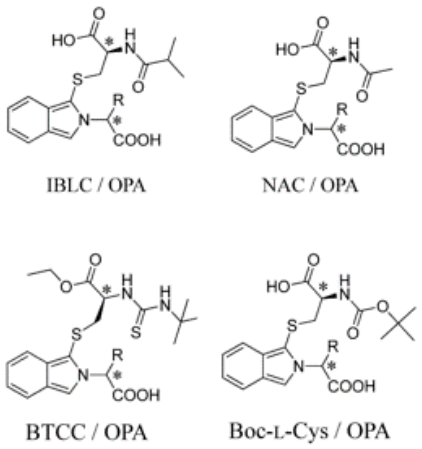

Figure 2-1 Amino acid derivatization with OPA and chiral thiols

This should facilitate trace detection by virtue of the high theoretical plate numbers for individual analytes. Such methods, however, are not practical due to the inherent enantiomeric impurity of the chiral derivatizing agents and have proven effective only for large concentrations of D-amino acids. Still, it is arguably useful to some extent and these methods are historically most popular and to this day are utilized for the investigation of free D-amino acids in animal tissue.[28,29,30,31]

One-dimensional methods that instead employ chiral stationary phases have also been applied for the determination of free D-amino acids in mammals. Limited reports have determined high concentrations of D-asp and D-ser in mammalian tissues.[32,33] In these reports, amino acids were derivatized with fluorescent reagents and analyzed on π-complex type stationary phases (i.e. dinitrobenzoyl phenylglycine ionically bonded to aminopropyl silica), a method found suitable for the two aforementioned analytes. Recently, Desai et al. reported a method employing one chromatographic chiral dimension in conjunction with atmospheric pressure chemical ionization mass spectrometry for the selective enantioseparation of 19 pure amino acids.[34] One

dimensional techniques involving chiral stationary phases typically lack the separation efficiency to effectively determine low concentrations. Additionally, the one dimensional techniques discussed here do nothing to minimize the inherent interferences of complex biological matrices.

2.1.2 Two dimensional HPLC methods

As an alternative, two-dimensional HPLC (LC/LC) methods have been preferentially implemented for trace determination of free amino acids. Various two dimensional methods have been established as highly selective and sensitive techniques capable of overcoming the complications associated with real biological matrices containing innumerable interferences. Multidimensional methods can be categorized into three general classes: directly coupled columns, comprehensive column switching, and heart-cutting.[35] As the name suggests, directly coupled column chromatography employs orthogonal phases, but one mobile phase must provide the desired separation on both columns, an inherent disadvantage.[35] Heart-cutting LC/LC allows the user to selectively resolve components of a specific effluent band from the first dimension on an orthogonal phase in the second dimension, whereas in comprehensive LC/LC the entire sample passes through both phases in succession. An essential advantage of these systems over one dimensional methods is the exponential increase in peak capacity, a hugely important feature in the analysis of biological samples. Heart-cutting instrumentation for biological samples proves immensely valuable when coupling an achiral dimension to a chiral dimension for subsequent separation of important enantiomers. Additionally, heart-cutting techniques allow users to individually optimize separation conditions in each dimension before joining them to work in succession (of course, the operating modes must be compatible). Finally, heart-cutting intrinsically preserves the integrity of the chiral column because only relatively pure effluent bands are directed to the second dimension.

Important considerations for developing methods in heart-cutting achiral-chiral chromatography are careful selection of the stationary phases, mobile phase compatibility, and miscibility of the matrix.[35]

In 1991, Armstrong et al. set a precedent for achiral-chiral heart-cutting methodologies for amino acid analysis as a system was developed for the determination of amino acid enantiomers in human urine using an ODS column coupled to a chiral crown ether, Crownpak CR(+), column.[36] This study inevitably prompted further inquiry into D-amino acid levels in various physiological fluids including human plasma, cerebrospinal fluid, and amniotic fluid.[37] A similar configuration was used to investigate the factors that controlled D-amino acid levels in laboratory rodents; age, pregnancy, diet, cancer, and sex all affected D-amino acid concentrations.[38] The chiral columns used for these studies were either chiral crown ethers, napthylethyl carbamoyl β-cyclodextrin, or native β-cyclodextrin. Pre or post-column derivatization for fluorescence detection was necessary as discussed in previous studies.[36-37] In these studies, large amounts of D-amino acids were found in the urine of rodents; in human physiological fluids, large amounts of D-trp, D-tyr, and D-phe were found in urine and plasma.

2D-HPLC procedures began to emerge for the determination of D-amino acids in mammalian tissues, and small levels of D-ser and D-asp were isolated from mice containing various D-amino acid oxidase activities.[39] In 2000, work from Inoue et al. described a 2D method for the determination of minute amounts of D-leu.[40] A more comprehensive narrative of 2D-HPLC methods for similar investigations can be found in review work from Miyoshi et al. in 2012.[28] Achiral-chiral multidimensional chromatography remains the best way to separate chiral analytes from natural interferences in biological matrices of increasing complexity.

2.1.3 Column technology: superficially porous particles

The search for improved separation methods has also included the recent development of superficially porous particles (SPP) for HPLC stationary phases. The advent of SPP's for achiral separations demonstrated efficiencies comparable to sub-2 μm fully porous particles (FPP) without the need for modified instrumentation to accommodate the ultrahigh pressures associated with smaller FPP's.[41-42] The performance of SPP columns in reverse phase separations has been evaluated extensively in empirical and theoretical studies.[43-44] For a typical C18 SPP separation, vastly improved efficiencies can be attributed to measurable reductions in all contributions to band broadening (i.e. eddy diffusion, longitudinal diffusion, and stationary phase mass transfer).[44-45] The manifestation of the decreased eddy diffusion contribution can generally be attributed to improved packing of the stationary phase as SPP particles tend to exhibit greater bed homogeneity from wall to center for a typical well packed column.[45-46] Additionally, the porous shell surrounding the solid core intrinsically limits trans-particle diffusion, lowering the mass transfer contributions to band broadening for large analytes with small diffusion coefficients and small analytes with slow adsorption-desorption kinetics.[41,43,45,47-48]

In more specialized branches of chromatography, namely chiral chromatography, the benefits of SPPs are only more recently explored. Initial applications of chiral stationary phases (CSPs) revealed a significant decrease in selectivity for polysaccharide coated SPPs compared with the analogous FPP columns.[49-50] More recently, Spudeit et al. presented brush type chiral selectors covalently bonded to SPPs with higher selector load per unit surface area; this along with substantial improvement in column efficiency for the CSP on SPP resulted in improved chiral separations with no loss in enantiomeric selectivity.[51] Perhaps the most comprehensive empirical examination of brush-type CSPs

on SPPs was recently demonstrated by Patel et al. in work presenting remarkably successful ultrafast chiral separations with efficiencies comparable to those typically seen for capillary electrophoretic separations.[45] The application of novel SPP column technology presented in this work serves two foremost points: (1) greatly enhanced detection of trace amino acids in the first dimension and (2) decreased analysis time in the second dimension without sacrificing enantiomeric selectivity, thus reducing band broadening and facilitating trace detection of the more retained D-enantiomer.

By virtue of the early investigations into D-amino acids in mammalian samples, the scientific community had unambiguous proof that these isomers were present. Questions regarding their ubiquity and physiological function were prompted; inquiries probing their biological activity elucidated the role of certain free D-amino acids, creating increased demand for a more comprehensive catalog as their importance was realized. More advanced methodologies for their isolation and detection naturally must be addressed. The work presented in this thesis provides an unambiguous method for the baseline determination of D-amino acids using advanced achiral-chiral heart-cutting two dimensional liquid chromatography. Superficially porous phases were applied to resolve the naturally occurring amino acids in the first dimension and the enantiomers of 11 selected amino acids in the second. Regional amino acid distributions were examined for the hippocampus and cortex of six laboratory mice. Additionally, here is presented the first comparison of total amino acid levels and D-isomer levels between non-perfused and perfused brain tissue (i.e. tissue for which the blood has been completely flushed).

2.2 Physiological Importance of D-Amino Acids

2.2.1 D-serine

Naturally, as detection of D-amino acids became feasible after maturation of pertinent analytical methods, the question of *why* they were relevant needed to be addressed. Techniques to distinguish the formerly overlooked D-isomers of amino acids initially revealed substantial quantities of D-serine and D-aspartate in particular. Now, these two amino acids are certainly the most thoroughly investigated and their regional distribution and biological activity are fairly well understood.[52] D-serine in particular has been most extensively studied. Free D-serine localizes predominantly in the mammalian forebrain, specifically in the cerebral cortex, hippocampus, and striatum with highest concentrations found in regions enriched in N-methyl-D-aspartate receptors.[26,30,53-54] D-serine, like other free D-amino acids, is acquired through diet, gastrointestinal bacteria, and liberation from metabolically stable proteins.[52] One implication of D-serine's immense importance in CNS function is the recent elucidation of its biosynthesis via enzymatic conversion of L-serine by serine racemase.[55] The close correlation between anatomical distribution of D-serine and the regional variation of the N-methyl-D-aspartate receptor (NMDAr) suggests a functional relationship. In fact, it is now well known that D-serine is an intrinsic co-agonist of the NMDAr, occupying the binding site once believed to be activated strictly by glycine.[56] In 2000, Mothet et al. demonstrated that D-serine is an endogenous ligand for the glycine binding site of the receptor with an even greater affinity than glycine.[56] Depletion of endogenous D-serine greatly attenuates functionality of the receptor, diminishing synaptic transmission and potentiating development of associated neurodegenerative disorders, namely schizophrenia and epilepsy.[57] As such, D-amino acids become clinically relevant as potential drugs. In fact, D-serine as a drug for

schizophrenia has had some success in clinical trials.[58] The importance of developing a more comprehensive catalog for D-amino acids in mammals is evident yet far from completion. Discoveries in this capacity provide clinicians and diagnosticians new insight into the implication of D-amino acid biomarkers in disease with potential treatment options.

2.2.2 D-Leucine

Accordingly, the investigation probed in this thesis work arose from the idea that D-leucine could be used as a suitable drug for epilepsy. Because there are so many cases of recurrent seizures that are not appropriately treated, a metabolism based therapy utilizing ketogenic diets has recently been revisited as a viable option.[59] L-leucine is a ketogenic amino acid; its degradation products include acetoacetate and β-hydroxybutarate, both of which fall into the class of anticonvulsants.[60] The issue with L-leucine as a treatment option is not in its inability to protect against seizure activity.[61] Elevated L-leucine and other branched chain amino acids have been linked to abnormal development and can actually induce seizures.[61] Alternatively, D-leucine remarkably has shown potent anti-seizure effects in clinical research conducted by Hartman, et al.[61] D-leucine is attained through diet and gastrointestinal bacteria and currently has no known physiological role in mammals. The fact that it shows remarkable anti-seizure effects in patients clearly demonstrates the importance of finding trends in D-amino acid levels in normal and diseased tissue. Thus, here we present an unambiguous method for the determination of D-amino acid levels in neurological tissue; work undertaken towards the advancement of a more comprehensive understanding of free amino acids in mammals.

Chapter 3

DETERMINATION OF TOTAL AND D-AMINO ACID CONCENTRATION BY ACHIRAL-CHIRAL TWO DIMENSIONAL LIQUID CHROMATOGRAPHY

3.1 Experimental

3.1.1 Materials

Natural amino acid racemates and all derivatization reagents, fluorenylmethyloxycarbonyl chloride, adamantadine hydrochloride, and boric acid, were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile and methanol were from Sigma-Aldrich. A Milli-Q water system (Millipore, Bedford, MA) was used to prepare ultrapure deionized water (18.2 MΩcm) for HPLC buffers. The 2.7 µm superficially porous silica particles were provided by Agilent Technologies (Wilmington, DE), as were the first dimension C18 columns (Poroshell 120 EC-C18, 4.6 x 150 mm i.d. 2.7 µm particles). Teicoplanin was covalently attached to the provided silica particles as described by Armstrong et al.[62] and the stationary phase was slurry packed into a 4.6 x 100 mm i.d stainless steel column (IDEX Health and Science, Oak Harbor, WA) as described by Patel et al.[45]

3.1.2 Derivatization of amino acid standards

Stock solutions of DL-amino acid standards were prepared with deionized water at concentrations of 0.03 M, with the exception of tryptophan and tyrosine which were prepared at concentrations of 0.01 M and 0.0025 M respectively. The standards were stored at 4°C while not in use. Borate buffer was prepared by mixing 25 mL 0.8 M boric acid in 0.8 M potassium chloride, adjusting the pH to 9 with 0.8 M NaOH, and thereafter diluting to 50 mL with deionized water. The stock FMOC solution was prepared by

dissolving 0.13 g in 5 mL acetonitrile (0.1 M). The stock adamantadine solution was prepared by dissolving 0.6 g in 5 mL of 1:1 acetonitrile: water (0.8 M). Amino acid standards were derivatized by adding directly to the autosampler vial, reagents in the following order: 50 µL amino acid stock solution, 400 µL borate buffer, 500 µL acetonitrile, 50 µL FMOC reagent. After twenty minutes, the reaction was stopped through the addition of 50 µL adamantadine reagent.

3.1.3 Mice brain non-perfused tissue

Male FVB/NJ mice (Jackson Laboratory, Bar Harbor, ME) aged 5-6 weeks old, weighing 25-30 g were used in all experiments. Mice were housed in groups of 4-5 in a cage under strictly controlled laboratory conditions with an artificial 12 hour light/dark cycle. A nutritionally-balanced rodent chow diet (Teklad Global 2018SX, Madison, WI) and tap water were continuously available. Animals were used in the study after at least one week of acclimatization. The study was carried out under experimental protocols approved by the ACUC. All efforts were made to minimize animal suffering.

Mice were sacrificed by cervical dislocation (to prevent pre and postsynaptic effects of anesthesia). Surgical scissors were used to remove the head. Using the scissors to make a midline incision, the skin was flipped over the eyes to free the skull and a small incision was made on the top of the skull from the caudal part at the point of the parietal bone, being careful to not cut through the brain. A firm cut was made through the most anterior part of skull. One side of the parietal bone was shifted before being separated from the remainder of the skull and this process was repeated bilaterally. Once the brain was released from the meninges, the curved pattern forceps were moved under the anterior part of the brain, which was then tilted smoothly upward. The forceps were used to incise the cranial and optic nerves, and the brain was then lightly lifted out of the skull. The brain was placed dorsal side up. The hindbrain was identified and a sagittal

incision was made to separate the two hemispheres (Figure 3-1). The hippocampus and cortex were then separated under a dissecting microscope.

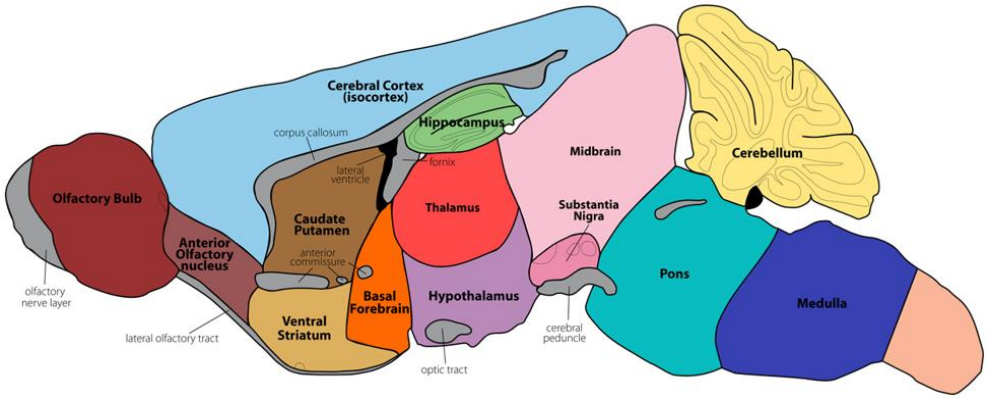

Figure 3-1 Sagittal plane view of mouse brain[63]

3.1.4 Mice brain perfused tissue

Mice were deeply anesthetized with carbon dioxide. A midline incision was made at the thoracic costal margin, followed by exposure of the peritoneal contents. A small incision was made at the xyphoid process and continued caudally. The sternum was opened broadly to visualize the heart and to incise the right atrium. Heparin/saline (APP Pharmaceuticals, LLC 1,000 USP Units/mL, Schaumburg, IL) and a blood collection set (BD Vacutainer, Four Oaks, NC) were injected using a 25 gauge butterfly needle 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 fluid exiting the mouse 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. Mice were then dissected according to the aforementioned procedure.

3.1.5 Free amino acid extraction

To all hippocampus and cortex samples was added 100 µL of an internal standard solution of norleucine (8.38 mM in DI water). The samples were homogenized in 1 mL of 0.1 N perchloric acid for 10 seconds (3 pulses) using a Q-Sonica CL-18 probe (Newtown, CT). The samples were maintained around 4 °C during ultrasonication. The homogenate was centrifuged at 13,000 RPM for 20 minutes at 4 °C and the supernatant was removed. 100 µL aliquots of the extract were subsequently derivatized in autosampler vials according to the procedure described for amino acid standards (*vide supra*). Brain material and extract were stored at -80 °C when not in immediate use.

3.1.6 HPLC instrumentation and method

The two dimensional chromatographic system used in the analysis was constructed through the conjunction 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). The system was equipped with heart-cutting capability through the incorporation of a Rheodyne 7000 six port stream switching valve (Rohnert Park, CA). A C18 column supplied by Agilent was used in combination with a chiral column synthesized and packed in-house. First dimension signal monitoring was done using ChemStation software from Agilent.

The first and second dimension conditions for separations were developed independently and thereafter tuned to work in series. The reverse phase HPLC gradient for individual amino acid isolation was performed on the Poroshell 120 EC-C18 with dimensions of 4.6 x 150 mm packed with 2.7 µm superficially porous particles. Mobile

phase A was 20 mM $H_2PO_4^-$ buffer adjusted to pH 2.5 with H_3PO_4; mobile phase B was acetonitrile. The gradient method began with 5% B (0-2 min) followed by a linear ramp from 15-80% B (2.01-35 min) then 80-95%B (35-38 min). Finally, the gradient concluded with a 2 minute ramp down to 5% B. The flow rate was 0.75 mL/min. With the exception of the co-eluting asparagine/glutamine peak and isoleucine/phenylalanine peak, this method successfully resolved the remaining amino acids of interest. A second achiral method was employed for the resolution of these four co-eluting amino acids. The two achiral reverse phase methods are shown comparatively in Figure 3-2. For the second reverse phase gradient, mobile phase A was 0.05 M sodium acetate; mobile phase B was a 23/22 (v/v) mixture of 0.1 M sodium acetate/acetonitrile. The gradient method began with a ramp from 30-37% B (0-3.75 min) followed by a ramp from 37-73% B (3.75-26.25 min) and finally brought to 100% B over the concluding 5 min. The diode array detector (DAD) monitored signals at 254 nm and the detector outlet was connected to the six port switching valve. Selected effluent bands could then be manually redirected to the second dimension column with a stream switch injection, typically lasting approximately 0.1 seconds (Figure 3-3). The resolution of the amino acid enantiomers was achieved on the teicoplanin column prepared in-house using an isocratic reverse phase method specific to the amino acid of interest.

Offline derivatization of amino acids was carried out using a well-studied and reliable method utilizing 9-fluorenylmehtlyoxycarbonyl chloride,[64] a reactive fluorescent tag for potentiation of detection signals, a particularly important feature for trace detection in the both dimensions. The fluorometric detection of FMOC-amino acids was conducted at an excitation wavelength of 254 nm and an emission wavelength of 313 nm. A representative separation of derivatized standards in the first dimension as well as the resolution of L and D enantiomers from heart-cut effluent bands is depicted in Figure 3-4.

Fractions of the eluting peak were manually introduced onto the enantioselective teicoplanin column for rapid chiral resolution. The chromatographic parameters for the enantioseparation of amino acid standards are outlined in Table 3-1. All chiral separations were easily achieved using the aforementioned house prepared teicoplanin column. The greatest advantage of implementing a SPP based CSP for this work proved to be the short analysis time and high efficiency gains without a sacrifice in selectivity between enantiomers; a comprehensive characterization of the column efficiency and performance can be found described by Patel et al.[45]

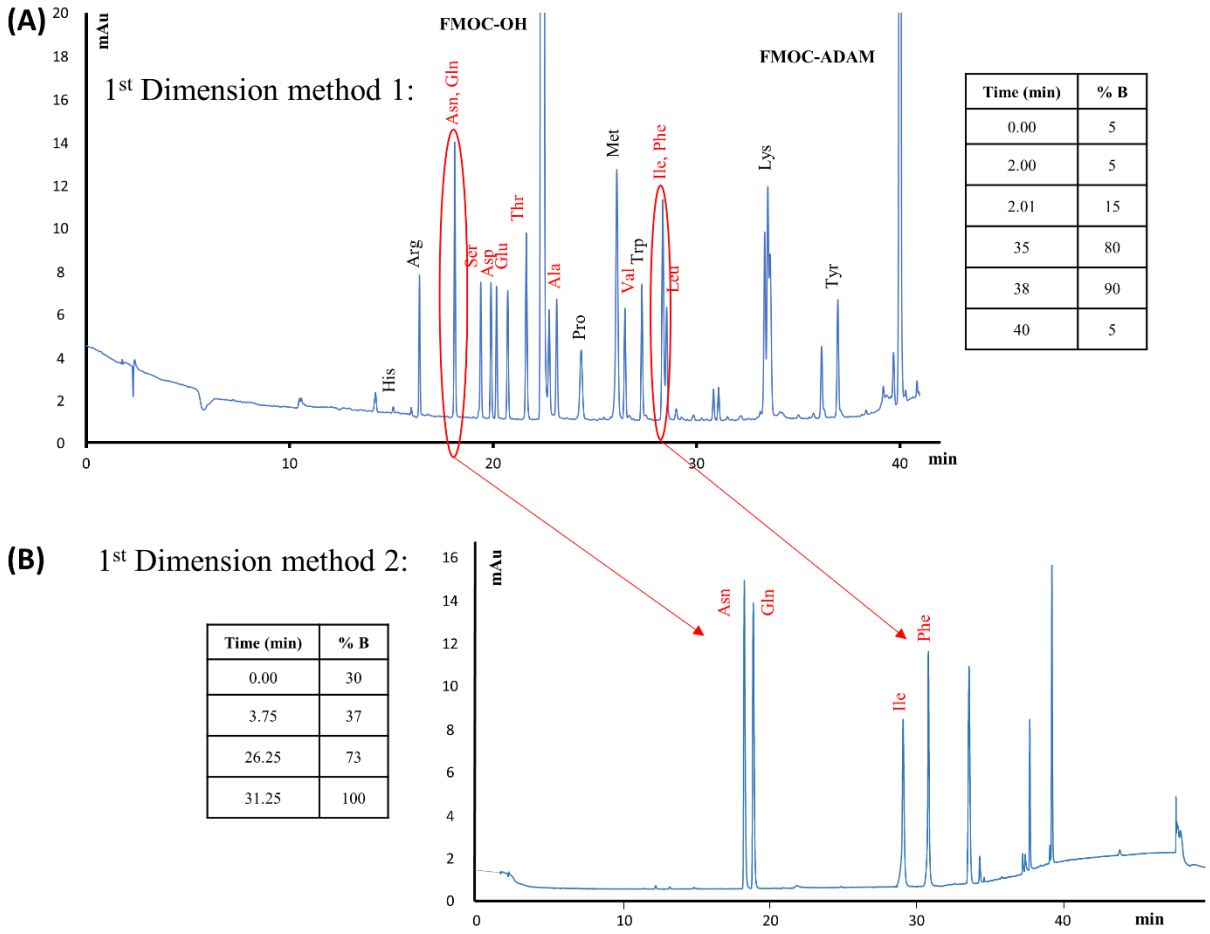

Figure 3-2 Achiral separation of amino acid standards on Agilent Poroshell EC-120 C18 (2.7 µm superficially porous particles, 4.6 x 150 mm i.d.) at 0.75 mL/min. Both gradients were run at ambient conditions. (A) Reverse phase gradient with MPA = 20 mM $H_2PO_4^-$ buffer adjusted to pH 2.5 with H_3PO_4; MPB = MeCN. (B) Reverse phase gradient with MPA = 0.5 M NaOAc; MPB = 23/22 (v/v) 0.1 M NaOac/MeCN.

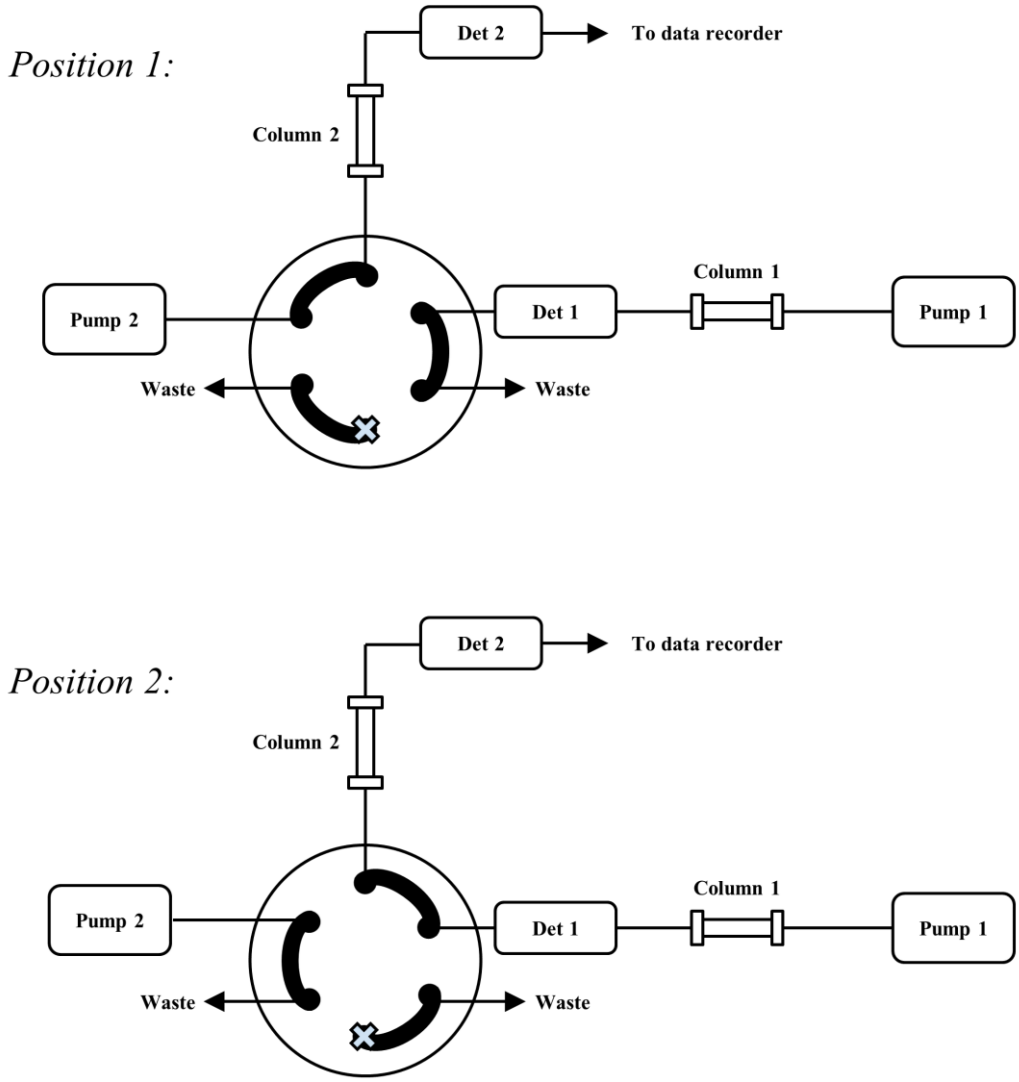

Figure 3-3 Flow diagram of the implemented six port stream switching valve. For normal operation during the separation of achiral analytes the valve remained in position 1. As effluent from the first column required redirection onto the second dimension, a fast and manual change was made to position 2 and immediately back to position 1.

3.2 Results and Discussion

3.2.1 Results

The total amino acid concentrations and D-amino acid percentages were examined separately from 12 specimens (i.e. the cortex and hippocampus of six mice). The ability to separate and quantify all of the naturally occurring amino acids in the first dimension was particularly challenging due to the inherent difficulties of dealing with biological samples. Extraction of the free amino acids using the outlined method sufficiently aided our efforts to accurately identify amino acid bands. Additionally, the first direct comparison of values from non-perfused and perfused brain tissue was here made. For accurate quantitation of total amino acid values, norleucine was added as an internal standard prior to homogenization of brain tissue and extraction of the free amino acid containing fluid. For each specimen, the extract was derivatized in triplicate and HPLC analysis was again done in triplicate. Figures 3-5 and 3-6 show representative two dimensional separations of brain extract samples.

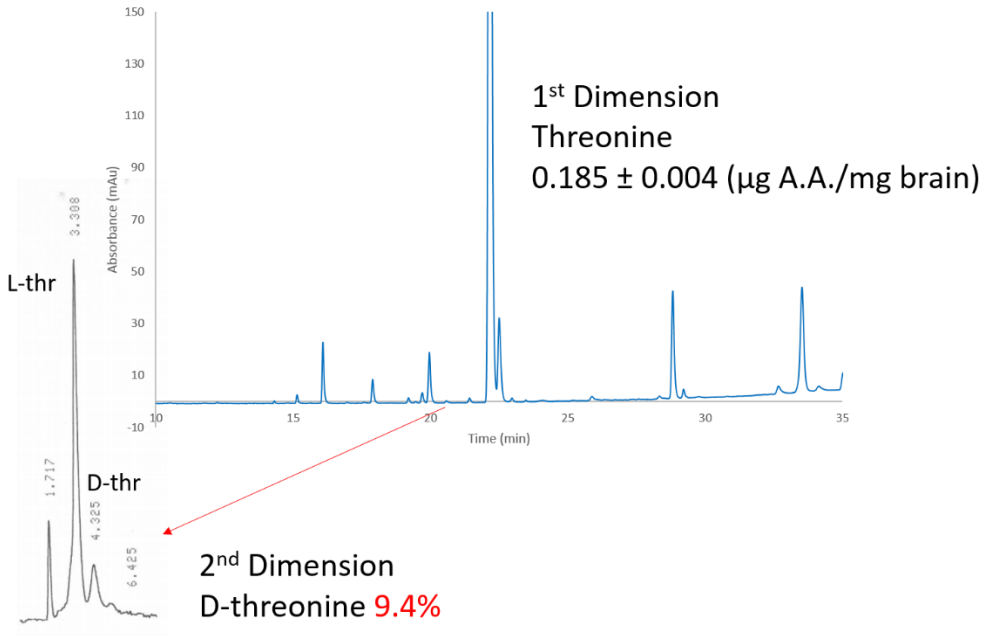

Figure 3-5 Representative two dimensional analysis of brain extract. Indicated threonine effluent band from a non-perfused cortex sample (NPC1) was redirected onto the second dimension column for enantiomeric resolution

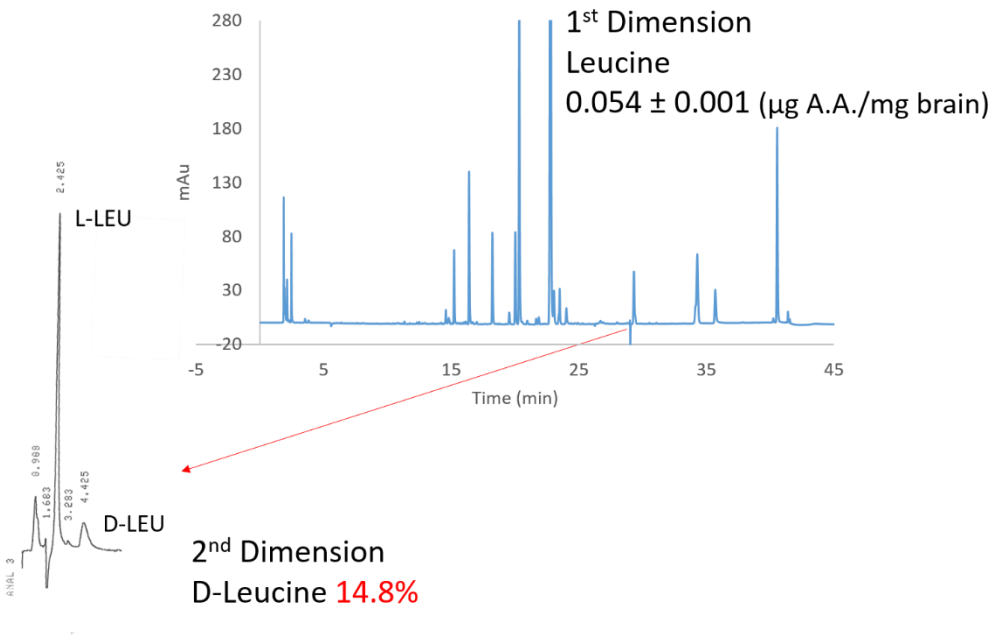

Figure 3-6 Representative two dimensional analysis of brain extract. Indicated leucine effluent band from a perfused cortex sample (PC2) was redirected onto the second dimension column for enantiomeric resolution

The results for total amino acid values for non-perfused and perfused tissues are summarized in Tables 3-2 and 3-3 respectively. Predictably, the three most abundant free amino acids in all studied brain tissue were found to be glutamic acid, glutamine, and aspartic acid. Due to their importance in neurotransmission pathways,[65-66] these amino acids have garnered the attention of the neurological community for some time. The high total levels we report here are consistent with previously reported results.[67-68] Free serine was also found in relatively high concentrations. The variation found in total amino acid content and %D values was relatively high. The factors effecting the %D amino acid have been previously studied by Armstrong, et al.[38] and could originate from a variety of sources. Unsurprisingly, when removing the blood (i.e. for perfused tissue), the variation

in total content was much lower. Generally, for a given amino acid, the total content found was greater for the hippocampus than for the cortex, with few exceptions (Figure 3-7). For both non-perfused tissue and perfused tissue, the amino acids occurring in the lowest total concentrations were leucine, asparagine, phenylalanine, and valine. Additionally, we found that the change in total amino acid content after tissue perfusion is relatively predictable, as seen from an examination of Figures 3-8 and 3-9. These plots show the average value for each amino acid in perfused tissue vs. the average value in non-perfused tissue. The fact that these scatter points fall on a straight line is rather interesting. Additionally, these plots also depict the vast improvement in variability when removing the blood from tissue samples. Individual %D values are presented in Figures 3-10 and 3-11. As a percentage, serine, asparagine, isoleucine, and phenylalanine were the most highly represented in their D isomeric form. However, as you convert these to total D concentration, the most abundant was serine in every case, with the exception of the perfused hippocampus. Glutamate was present only as the L-isomer in all cases. It is evident from a careful evaluation of Figures 3-12 through 3-15 that the blood introduces a variable that makes prediction of total free D-amino acid concentration relatively difficult. It also allows us to assess the importance of the D-amino acid as a neurological necessity: as this study presents the first direct comparison between tissue containing blood and tissue flushed of its blood, it is reasonable to assume that the D-amino acids seeing diminished occurrence with removal of the blood were, obviously, present in the blood itself rather than the neurological tissue. This could suggest that these were destined for excretion or metabolic processes rather than collection as neurotransmitters or developmental agents. The increased regularity and predictability of the free amino acid content and D-amino acid content for perfused tissues is in accordance with this inference. The five most highly represented D-amino acids in the perfused hippocampus

and perfused cortex were in both cases serine, asparagine, glutamine, alanine, and aspartic acid (Figures 3-10 and 3-11). D-leucine was not highly represented in the tissue from the examined mice, but as our collaborators have demonstrated, offers a potential treatment option for epileptic test subjects.

3.2.2 Discussion

L-glutamate was the most abundant amino acid for all tissue samples examined. I has been suggested that L-glutamate, L-aspartate, and D-serine are important neuromodulators of the NMDA glutamate receptor.[69] As previously mentioned, simultaneous binding of the agonist and co-agonist are required for its activity. The role of D-aspartate is not clarified, however it occurs in high concentrations in the nerve tissue of mammals during embryonic and fetal stages, in some cases exceeding the concentration of its enantiomer, suggesting its importance in neurogenesis and growth. D-aspartic acid also binds to the glutamate site of the NMDA receptor, an indication of its roll in neurotransmission. Glutamine, glutamate, and aspartate are also intimately involved in the γ-aminobutyric acid (GABA) neurotransmission system. Glial cells that receive glutamate released by pyramidal neurons convert the neurotransmitter to glutamine with the enzyme glutamine synthetase.[70] Subsequent release back to the neuron results in hydrolysis of the glutamine back to glutamate to replenish the pool. Glutamine is also a precursor to GABA itself.[71] D-alanine does not have such an elucidated role in mammals, though its presence in healthy and Alzheimer diseased neurological tissue has been evaluated.[72-73] Similarly, as yet, D-asparagine and D-leucine (along with the other amino acids quantified in this study) have no known physiological function in mammals. The necessity for advancement of physiological knowledge is evident and the diagnostic values of D-amino acids is surely recognized by neurological scientists and clinicians. The work provided here represents the first highly advanced analytical method for the

determination of trace amino acids and their D-isomers, and will surely prove useful in the field. Additionally, the data presented represents the first investigation into non-perfused brain tissue, providing neuroscientists a more informative account of the localization of amino acids. Thus, the contribution is fourfold: analytically, a progressive and unambiguous heart cutting method for the determination of D-amino acids was successfully implemented; more amino acids were analyzed in this work than have been so far investigated in a single study, and valuable base line concentration values have been presented for healthy laboratory mice; clinically, the human life sciences gained a valuable tool for elucidation of the physiological importance of the studied amino acids; finally, tissue for which the blood has been removed has been compared directly to tissue containing blood, an insightful advancement in amino acid neurochemistry.

3.2.3 Future Work

The total amino acid content and D-amino acid content were successfully determined for 11 analytes from the neurological tissue of 6 mice (each separated into hippocampus and cortex; 3 mice non-perfused and 3 mice perfused) and thus 12 samples. The statistical significance of the findings here require validation by an increased sample size. Thus, work is ongoing to double *n* by examining the neurological contents of an 6 additional mice.

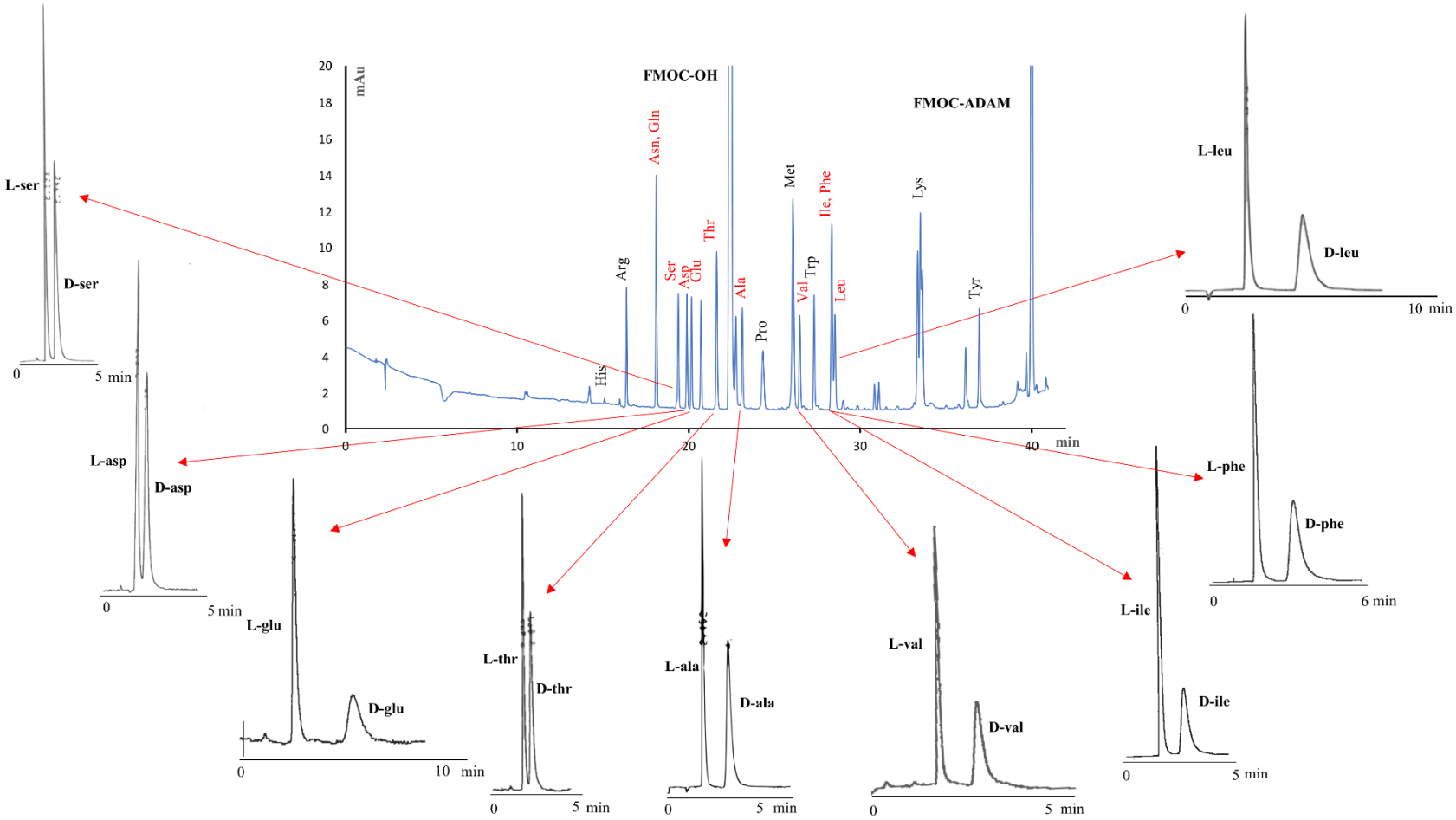

Figure 3-4 Reverse phase separation of derivatized amino acid standards in the first dimension with resolution of amino acid enantiomers from indicated effluent bands in the second dimension

Table 3-1 Chromatographic data and mobile phase conditions for FMOC-amino acid standards in second dimension

FMOC-Amino Acid	k_1	α	R_s	Mobile Phase
Leucine	2.02	2.18	2.7	60/40 0.1% TEAA (pH=4.1)/MeOH
Serine	2.01	1.60	1.8	70/30 0.1% TEAA (pH=4.1)/MeOH
Alanine	2.87	2.51	3.7	70/30 0.1% TEAA (pH=4.1)/MeOH
Aspartic Acid	2.34	1.49	1.4	70/30 0.1% TEAA (pH=4.1)/MeOH
Glutamic Acid	2.42	2.79	3.2	70/30 0.1% TEAA (pH=4.1)/MeOH
Threonine	1.29	1.57	1.4	70/30 0.1% TEAA (pH=4.1)/MeOH
Valine	1.78	1.93	2.5	60/40 0.1% TEAA (pH=4.1)/MeOH
Asparagine	1.36	1.61	1.5	60/40 0.1% TEAA (pH=4.1)/MeOH
Glutamine	1.37	2.48	2.4	60/40 0.1% TEAA (pH=4.1)/MeOH
Isoleucine	1.25	2.25	2.2	55/45 0.1% TEAA (pH=4.1)/MeOH
Phenylalanine	1.53	2.48	2.4	55/45 0.1% TEAA (pH=4.1)/MeOH

Table 3-2 Total amino acid content for non-perfused brain tissue

	NPC 1	**NPH 1**	**NPC 2**	**NPH 2**	**NPC 3**	**NPH 3**
	A.A. (µg/mg)	A.A. (µg/mg)	A.A. (µg/mg)	A.A. (µg/mg)	A.A. (µg/mg)	A.A. (µg/mg)
Leu	0.0320 ± 0.0004	0.14 ± 0.01	0.043 ± 0.001	0.20 ± 0.03	0.053 ± 0.001	0.059 ± 0.001
Ser	0.4860 ± 0.0002	0.602 ± 0.001	0.4480 ± 0.0001	0.519 ± 0.001	0.34 ± 0.02	0.339 ± 0.002
Ala	0.30 ± 0.01	0.5 ± 0.1	0.34 ± 0.01	0.45 ± 0.01	0.34 ± 0.01	0.51 ± 0.01
Asp	1.82 ± 0.02	1.58 ± 0.05	1.461 ± 0.002	1.07 ± 0.04	1.4 ± 0.1	0.97 ± 0.01
Thr	0.185 ± 0.004	0.227 ± 0.001	0.199 ± 0.002	0.31 ± 0.01	0.12 ± 0.01	0.238 ± 0.002
Glu	8.1 ± 0.8	9 ± 2	7.6 ± 0.1	5.8 ± 0.5	4.3 ± 0.2	4.0 ± 0.1
Val	0.12 ± 0.03	0.20 ± 0.01	0.087 ± 0.002	0.22 ± 0.01	0.034 ± 0.001	0.142 ± 0.001
Asn	0.062 ± 0.001	0.25 ± 0.03	0.091 ± 0.002	0.33 ± 0.01	0.033 ± 0.001	0.089 ± 0.001
Gln	2.51 ± 0.03	3.1 ± 0.4	2.22 ± 0.04	2.1 ± 0.1	2.1 ± 0.1	1.71 ± 0.01
Ile	0.025 ± 0.003	0.12 ± 0.02	0.029 ± 0.004	0.08 ± 0.01	0.0220 ± 0.0003	0.0330 ± 0.0003
Phe	0.05 ± 0.01	0.19 ± 0.04	0.05 ± 0.01	0.30 ± 0.02	0.054 ± 0.001	0.072 ± 0.001

Table 3-3 Total amino acid content for perfused tissue

	PC 1	PH 1	PC 2	PH 2	PC 3	PH 3
	A.A. (µg/mg)	A.A. (µg/mg)	A.A. (µg/mg)	A.A. (µg/mg)	A.A. (µg/mg)	A.A. (µg/mg)
Leu	0.048 ± 0.001	0.078 ± 0.001	0.054 ± 0.001	0.059 ± 0.001	0.054 ± 0.001	0.051 ± 0.001
Ser	0.18 ± 0.02	0.25 ± 0.01	0.237 ± 0.002	0.281 ± 0.002	0.25 ± 0.01	0.266 ± 0.003
Ala	0.29 ± 0.01	0.34 ± 0.02	0.313 ± 0.002	0.353 ± 0.001	0.377 ± 0.001	0.37 ± 0.01
Asp	1.11 ± 0.04	0.84 ± 0.01	1.22 ± 0.02	1.11 ± 0.01	1.40 ± 0.01	1.19 ± 0.01
Thr	0.063 ± 0.004	0.083 ± 0.003	0.08 ± 0.002	0.170 ± 0.002	0.104 ± 0.004	0.126 ± 0.001
Glu	4.8 ± 0.2	4.27 ± 0.03	5.0 ± 0.1	4.40 ± 0.05	4.98 ± 0.02	4.32 ± 0.03
Val	0.019 ± 0.001	0.048 ± 0.003	0.025 ± 0.001	0.114 ± 0.001	0.026 ± 0.001	0.0420 ± 0.0002
Asn	0.033 ± 0.001	0.099 ± 0.001	0.032 ± 0.001	0.0300 ± 0.0003	0.0330 ± 0.0003	0.1080 ± 0.0004
Gln	1.8 ± 0.1	1.584 ± 0.02	1.75 ± 0.03	1.68 ± 0.02	1.86 ± 0.02	1.73 ± 0.01
Ile	0.012 ± 0.001	0.0200 ± 0.0002	0.0120 ± 0.0002	0.019 ± 0.001	0.0220 ± 0.0004	0.0090 ± 0.0001
Phe	0.041 ± 0.002	0.0350 ± 0.0004	0.045 ± 0.001	0.042 ± 0.003	0.049 ± 0.001	0.0370 ± 0.0002

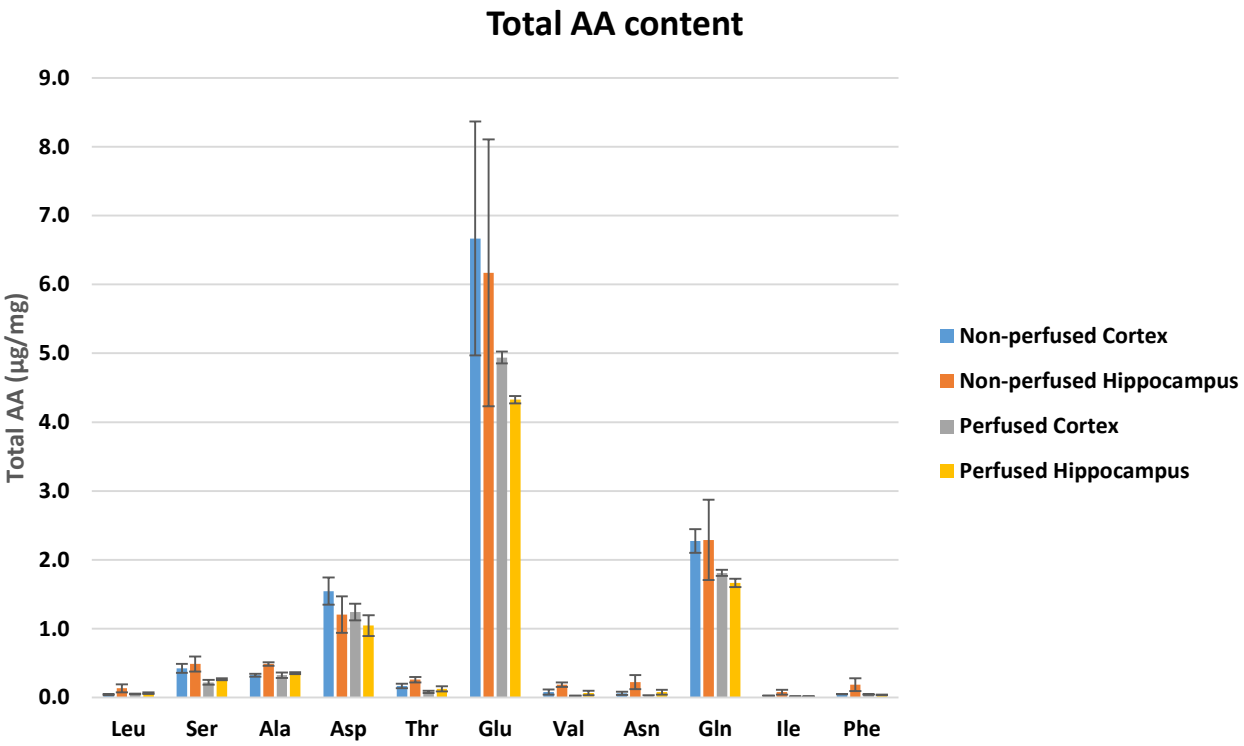

Figure 3-7 Comparison of average total amino acid content for each tissue type

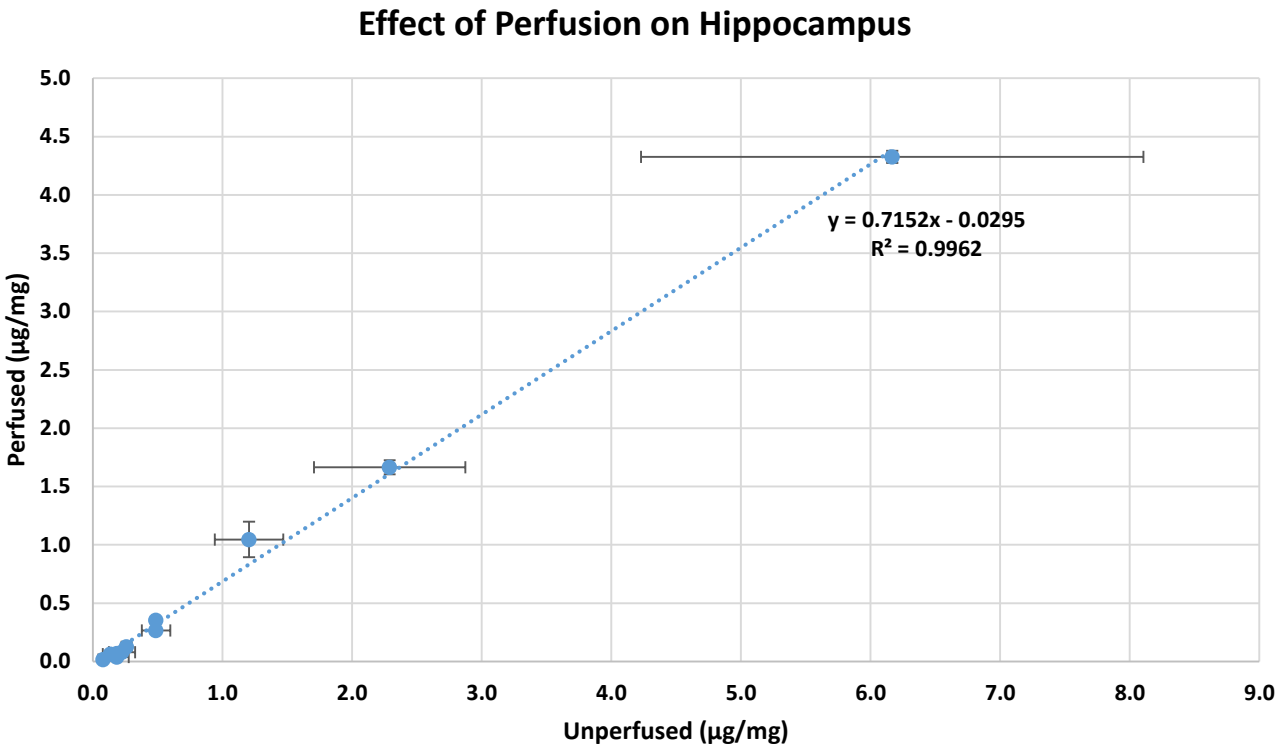

Figure 3-8 Effect of tissue perfusion on hippocampus. Each data point represents an individual amino acid.

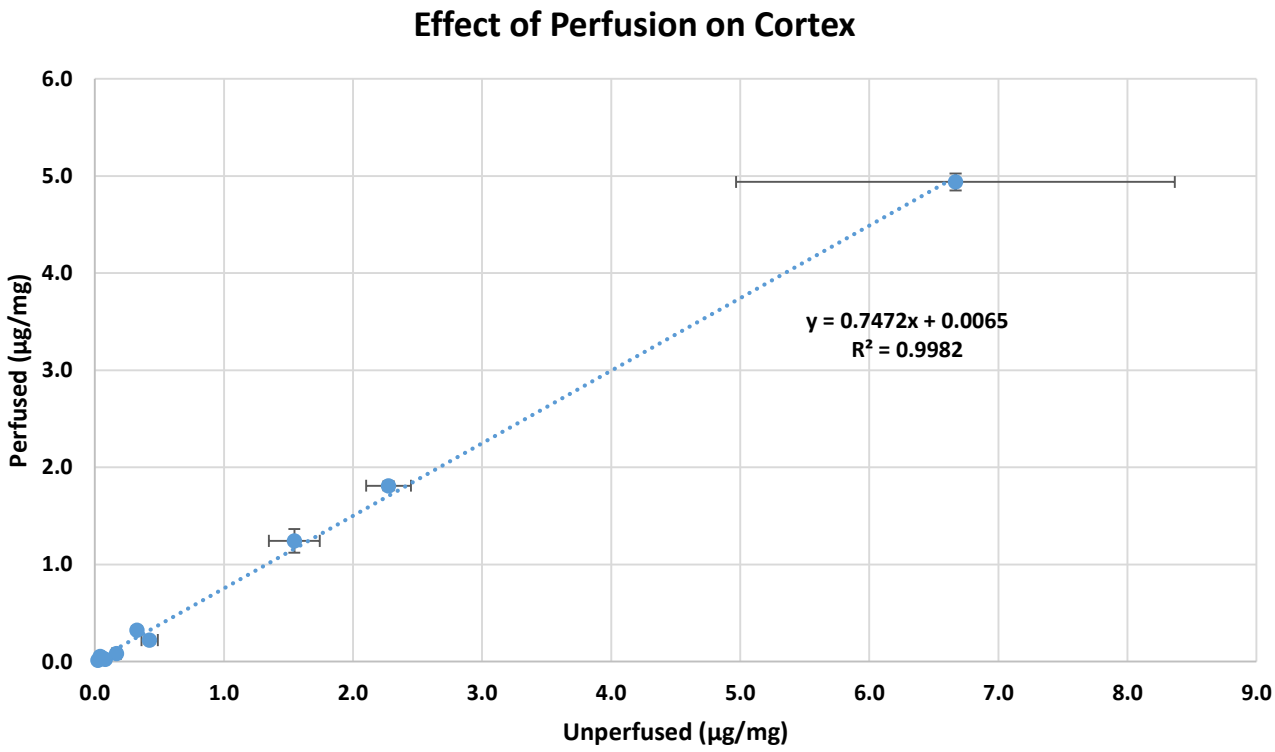

Figure 3-9 Effect of tissue perfusion on cortex. Each data point represents an individual amino acid.

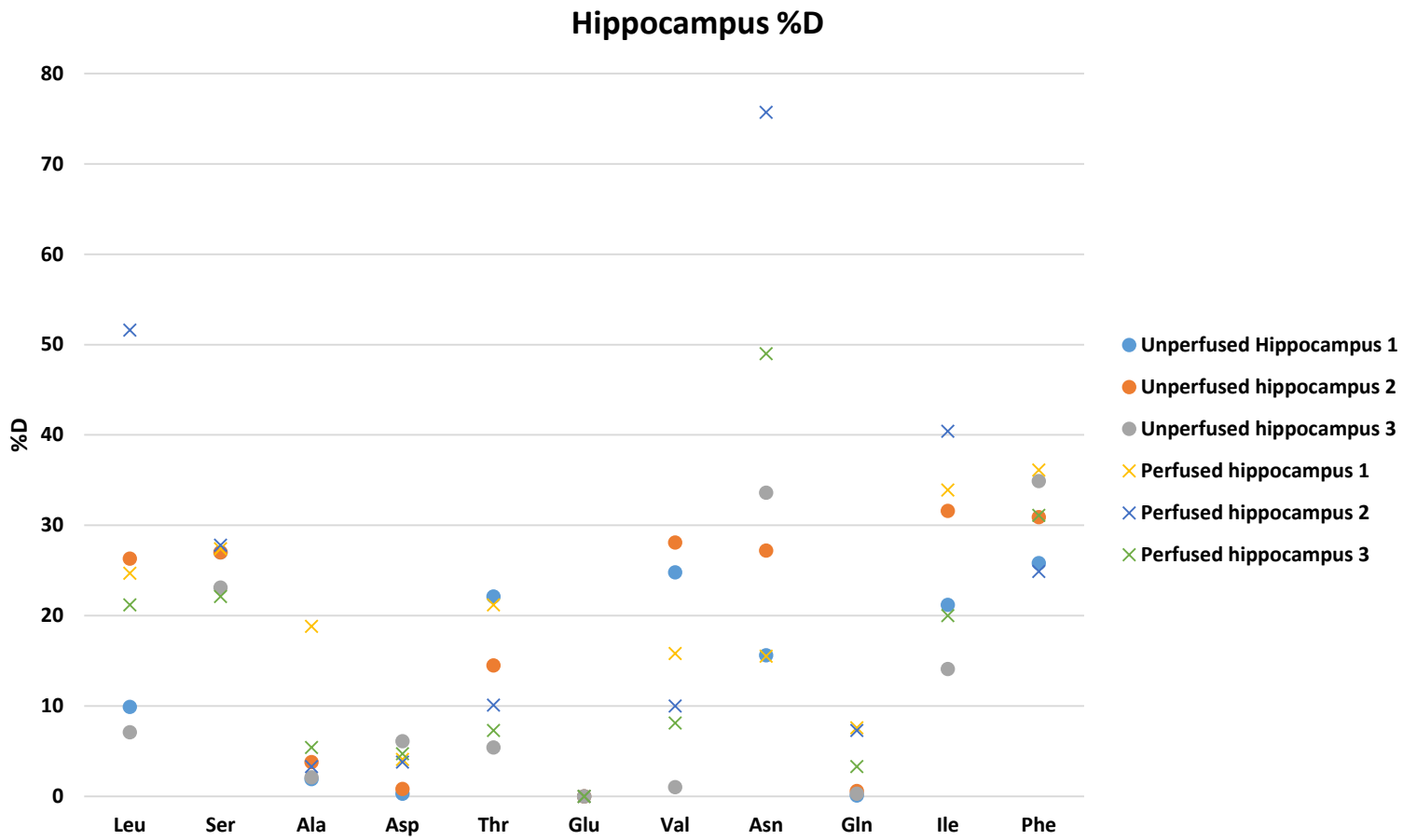

Figure 3-10 %D values for each amino acid from all hippocampus samples.

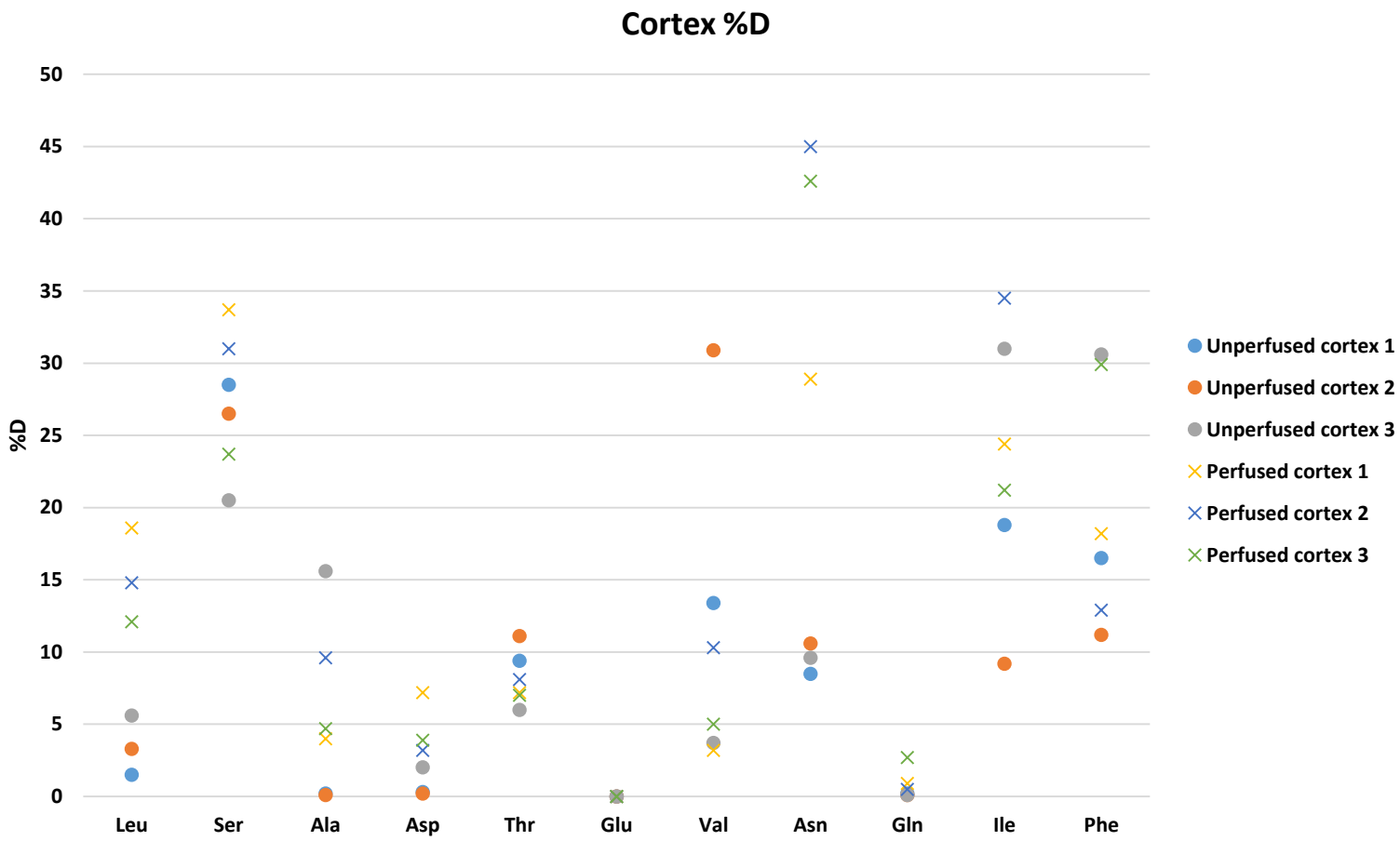

Figure 3-11 %D values for each amino acid from all cortex samples

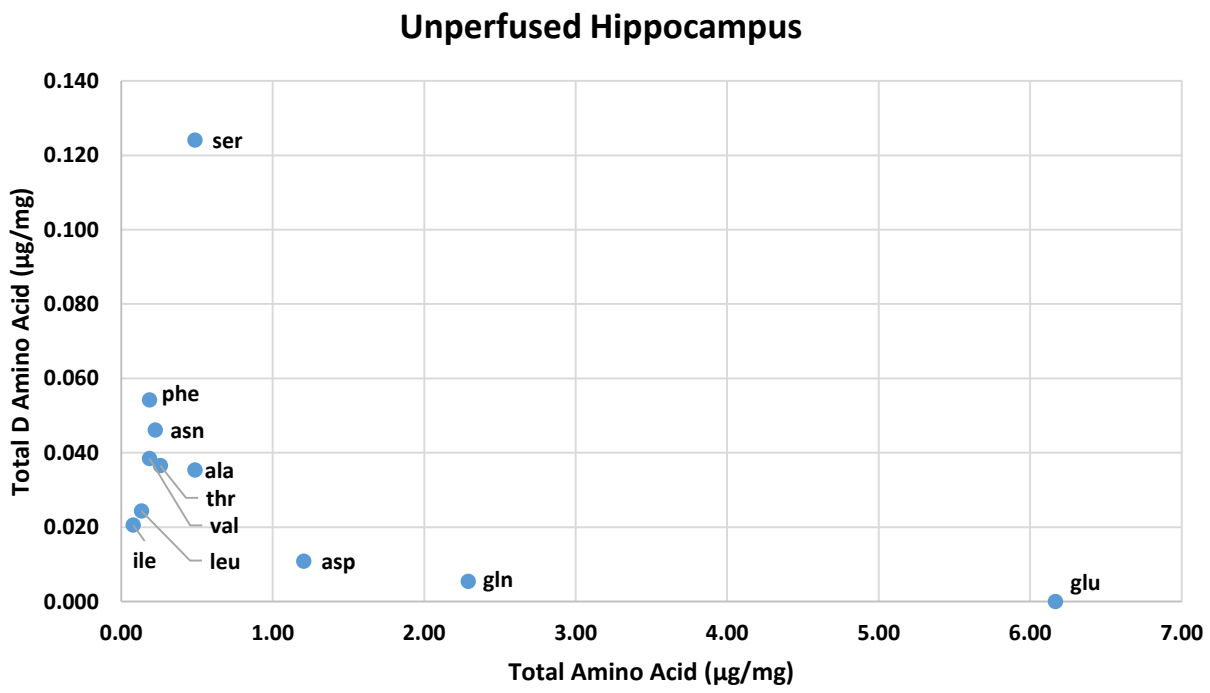

Figure 3-12 Average free D-amino acid concentration vs. average total free amino acid concentration for the non-perfused hippocampus samples

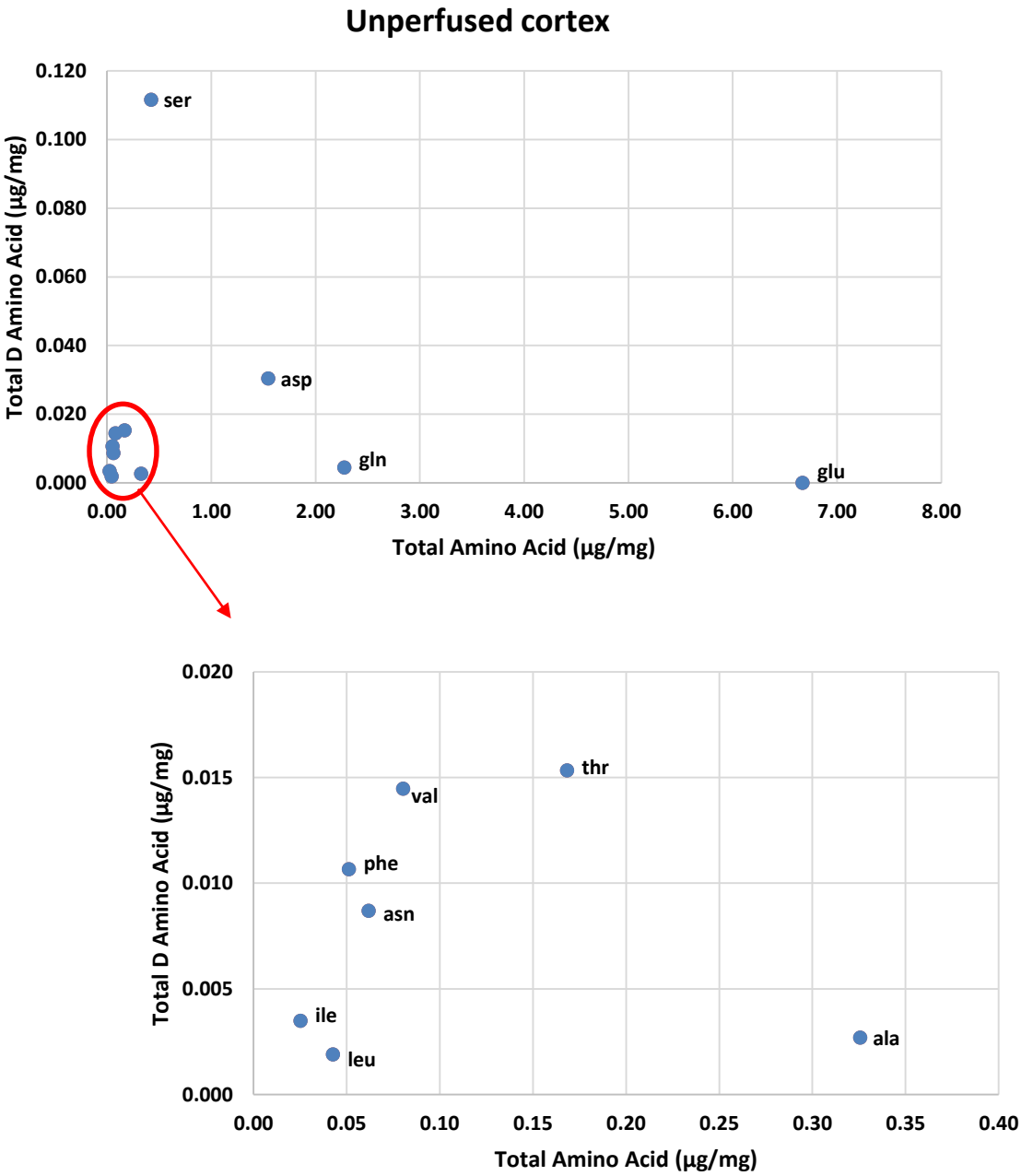

Figure 3-13 Average free D-amino acid concentration vs. average total free amino acid concentration for the non-perfused cortex samples

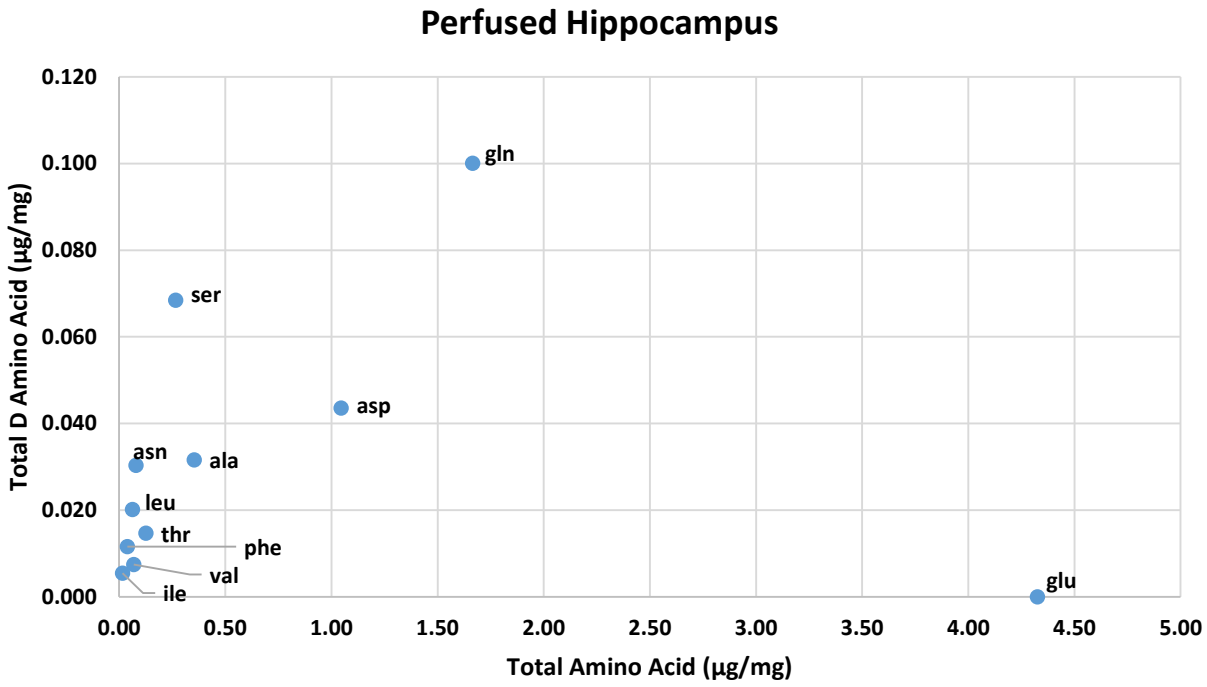

Figure 3-14 Average free D-amino acid concentration vs. average total free amino acid concentration for the perfused hippocampus samples

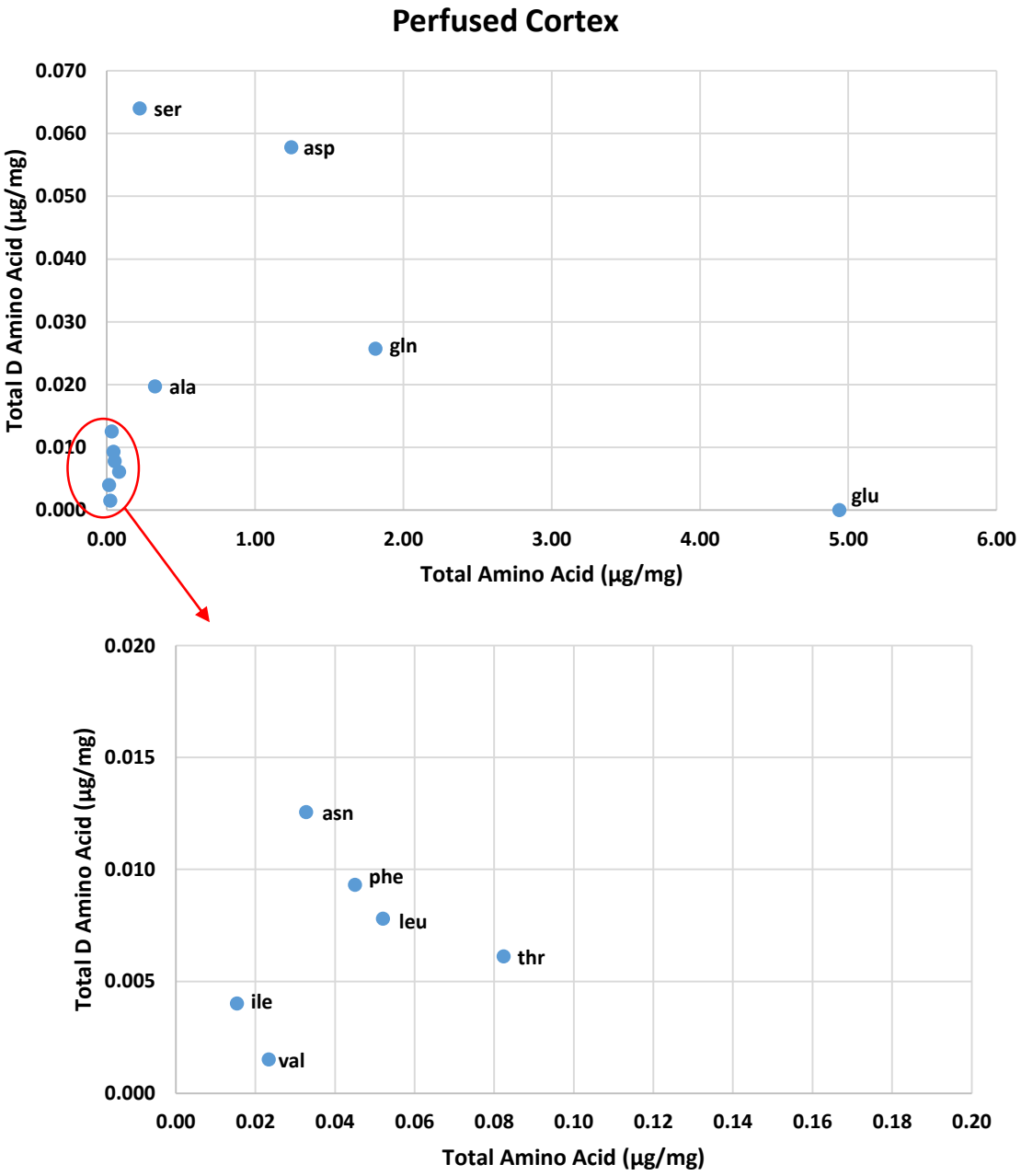

Figure 3-15 Average free D-amino acid concentration vs. average total free amino acid concentration for the perfused cortex samples

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Biographical Information

Curran Parpia was born in 1990 in Bedford, Texas into a family of scholars. In 2008, after graduating from Trinity HS in Euless, Texas, Curran left the state to pursue a degree in Biomedical Engineering at Purdue University. After one year attending Purdue, Curran returned to Texas despite seeing academic success, realizing that his heart was neither in his degree plan nor in West Lafayette, Indiana. In 2010, after his first year attending the University of Texas at Arlington as a chemistry major, Curran was awarded the Honors Undergraduate Research and Creative Activity grant for one summer of paid research in the Foss Laboratory, where he was exposed to organic synthesis. Curran graduated from the University of Texas at Arlington with a Bachelor's of Science in Biochemistry in May, 2012. After a direct transition into the graduate program, Curran still believed organic synthesis was the route he wished to pursue. However, as a Master's student, Curran found passion in analytical chemistry after topping the class of his future advisor, Dr. Daniel Armstrong. Curran greatly enjoyed the field of chromatography and separation science, with a tremendous admiration for his advisor Dr. Armstrong. In good health, Curran hopes to remain on the path to success and complete his PhD in Analytical Chemistry.