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Quantitative Studies of Amyloidogenic Protein Residue Interaction Networks and Abnormal Ammonia Metabolism in Neurotoxicity and Disease

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ABSTRACT

Quantitative Studies of Amyloidogenic Protein Residue Interaction Networks and Abnormal Ammonia Metabolism in Neurotoxicity and Disease

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Investigating similarities among neurological diseases can provide insight into disease processes. Two prominent commonalities of neurological diseases are the formation of amyloid deposits and altered ammonia and glutamate metabolism. Computational techniques were used to explore these processes in several neurological diseases. Residue interaction networks (RINs) abstract protein structure into a series of nodes (representing residues) and edges (representing connections between residues likely to interact). Analyzing the RINs of non-amyloid forms of amyloidogenic proteins for common network features revealed similarities not previously known. First, amyloidogenic variants of lysozyme were used to demonstrate the usefulness of RINs to the study of amyloidogenic proteins. Next, I compared RINs of amyloidogenic proteins with randomized control networks and a group of real protein controls and found similarities in network structures unique to amyloidogenic proteins. The use of 3D structure data and network structure data of amyloid-beta (1-42) (Abeta42) in a hydrophobic, membrane-mimicking solvent led to the identification of an interaction between Val24 and Ile31 as potentially involved in preventing Abeta aggregation. Since Abeta causes oxidative damage, since the ammonia

metabolism enzyme glutamine synthetase is particularly susceptible to oxidative damage, and since glutamate plays a central role in neuronal function, I expanded my research to include the study of ammonia and glutamate metabolism in neurological diseases. A computational model of the effects of the interactions between the amount of dietary protein and the activities of ammonia metabolism enzymes on blood and brain ammonia levels supports potentially important roles for these enzymes in the protection of neural function. Next, I reviewed the role of amino acid catabolism in Alzheimer's disease (AD). Common tissue pathology and the ability of memantine, an NMDA receptor antagonist, to relieve symptoms in patients and animal models of AD, major depressive disorder (MDD), and type 2 diabetes (T2D) further support a role for ammonia and glutamate metabolism in disease. Lastly, I found that single nucleotide polymorphisms (SNPs) in select ammonia metabolism genes are associated with these three diseases. The results presented in this dissertation demonstrate that investigating neurological diseases using computational approaches can provide great insight into the common underlying pathologies.

DEDICATION

I dedicate my dissertation to Wayne Birchfield, my grandfather. He is the inspiration for much of this research, and I strive to follow his example of integrity and dedication in both my professional and personal life.

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I would like to acknowledge my parents, Buck and Cindy Arch. My mom has been my "coach" since starting school, and I am thankful for her role in helping me achieve my academic goals. Buck has "swooped in" to save the day many times during my education. I would also like to thank my wife, Holly, for her encouragement and support throughout my graduate school experience.

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ABBREVIATIONS

α-Syn, alpha-synuclein

Abeta, amyloid-beta

Abeta40, amyloid-beta (1-40)

Abeta42, amyloid-beta (1-42)

ACMS, α-amino-β-carboxymuconate-ε-semialdehyde

AD, Alzheimer's disease

ADP, adenosine diphosphate

Arg2, arginase II

ATF4, activating transcription factor 4

ATP, adenosine triphosphate

BBB, blood-brain barrier

BCAA, branched chain amino acid

BCKDH, branched chain ketoacid dehydrogenase

BDNF, brain-derived neurotrophic factor

CHC, central hydrophobic cluster

CNS, central nervous system

CPS1, carbamoyl phosphate synthetase 1

CSF, cerebrospinal fluid

DFAM, family-based association for disease traits

E2F4, E2F transcription factor 4

eIF2α, eukaryotic initiation factor 2 alpha

ER, endoplasmic reticulum

ETS1, ETS proto-oncogene 1, transcription factor

FDG, fluorodeoxyglucose

FOXP3, forkhead box P3

GABA, gamma-aminobutyric acid

GCN2, general control nondepressible 2

GLS, glutaminase

GLUD, glutamate dehydrogenase

GLUL, glutamine synthetase, glutamate-ammonia ligase

GML, Graph Markup Language

GTP, guanosine triphosphate

GWAS, genome-wide association study

HE, hepatic encephalopathy

HFIP, hexafluoroisopropanol

hPrP, human prion protein

HWE, Hardy-Weinberg equilibrium

KP, kynurenine pathway

LB, Lewy body

LD, linkage disequilibrium

MAF, minor allele frequency

MCI, mild cognitive impairment

MCODE, molecular complex detection

MDD, major depressive disorder

mTOR, mammalian target of rapamycin

mTORC1, mammalian target of rapamycin complex 1

NAC, N-acetylcysteine

NAD, nicotinamide adenine dinucleotide

NCBI, National Center for Biotechnology Information

NGF, neural growth factor

NIA-LOAD, National Institute on Aging—Late Onset Alzheimer's Disease

NMDA, N-methyl-D-aspartate

OMIM, Online Mendelian Inheritance in Man

OTC, ornithine transcarbamoylase

PDB, Protein Data Bank

PERFECTOS-APE, predicting regulatory functional effect of SNPs by approximate p-value estimation

PGR, Protein Graph Repository

PI3K, phosphatidylinositol 3-kinase

PLINK, population-based linkage analyses software

PrP, prion protein

PrP^{C}, cellular form of prion protein

PrP^{SC}, infectious form of prion protein

QUIN, quinolinic acid

RIN, residue interaction network

RING, Residue Interaction Network Generator

RMSD, root mean square deviation

ROS, reactive oxygen species

SCOP, Structural Classification of Proteins

SDS, sodium dodecyl sulfate

SNARE, soluble NSF-attachment protein receptor

SNP, single nucleotide polymorphism

T2D, type 2 diabetes

TCA, tricarboxylic acid

TF, transcription factor

TFE, trifluoroethanol

TSE, transmissible spongiform encephalopathy

WT, wild type

CHAPTER 1

INTRODUCTION

Many Neurological Diseases Have Common Etiologies and Phenotypes

Neurological diseases are a diverse group of disorders, so researchers often study them using a reductionist approach. This view has successfully led to a large body of knowledge and successful treatments for some diseases. However, a broader approach that attempts to find similarities among some of the proposed causes of neurological disease could lead to novel and more broad-spectrum treatments. Improper protein folding and metabolic abnormalities are two of the proposed shared causes of neurological diseases and their symptoms. Improper protein folding results in the formation of insoluble protein deposits called amyloid plaques (Rambaran and Serpell 2008). Metabolic abnormalities are understandably diverse in neurological diseases due to the central role of metabolism in cellular function, but amino acid and ammonia metabolism stand out as having influential roles in many diseases. The overall goal of this dissertation is to use computational analyses to provide insight into potential molecular contributors to select neurological diseases by investigating abnormal ammonia metabolism and the residue interaction network structures of amyloidogenic proteins, two likely contributors to neurological diseases. I propose two guiding hypotheses for this series of studies.

(A) Amyloidogenic proteins have similarities in their residue interaction network structures that will provide insight into the formation of amyloid plaques.

(B) Altered ammonia metabolism and changes in the amount of dietary protein

under certain conditions affect neurological function and play causative roles in several neurological diseases.

These studies investigated possible contributing factors to neurological diseases and report commonalities in improper protein folding and abnormal metabolism. Studies of broad scope that attempt to find commonalities in the causes of neurological diseases could provide novel insight into possible treatments or preventions. This series of studies is organized into specific aims which address hypotheses **(A)** and **(B)**.

Specific Aim 1

To determine if amyloidogenic proteins have commonalities in their residue interaction networks and to determine how these network structures may affect amyloid-beta plaque formation when combined with 3D structural data. This aim addresses hypothesis **(A)**.

Amyloidogenic Proteins Are Involved in Many Neurological Diseases

Amyloidogenic proteins are polypeptides that can form amyloid plaques (Knowles et al. 2014). Amyloid plaques are associated with a variety of diseases ranging from neurological disorders (Glenner and Wong 1984; Hardy and Higgins 1992) to gastrointestinal disease (Granel et al. 2002). The symptoms of the diseases depend on the tissue in which the amyloids are deposited. There are dozens of different amyloid diseases caused by dozens of different proteins (Knowles et al. 2014). The structure of amyloid plaques of a variety of proteins consists of insoluble stacks of cross-beta sheets (Sunde et al. 1997). There is little known similarity in native structure, sequence, or function of

amyloidogenic proteins in monomeric form (Knowles et al. 2014). Several amyloid diseases (amyloidoses) are characterized by deposition of plaques in the central nervous system (CNS), leading to a variety of different but related symptoms. Amyloidoses of the CNS include amyloid-beta (Abeta) in Alzheimer's disease (Glenner and Wong 1984), alpha-synuclein (α-syn) in Parkinson's disease (Spillantini et al. 1997), and human prion protein (hPrP) in transmissible spongiform encephalopathies (TSEs) (Kretzschmar et al. 1986; Nizhnikov et al. 2016).

Amyloid-Beta and Alzheimer's Disease. The amyloid hypothesis for Alzheimer's disease was first proposed in 1992 by Hardy and Higgins (Hardy and Higgins 1992), but it has its roots in the first studies of purified Abeta (Glenner and Wong 1984). The hypothesis states that aggregates of the Abeta peptide are responsible for the cognitive decline and cellular damage observed in Alzheimer's disease. Since then, the amyloid hypothesis has been thoroughly investigated with data both supporting and refuting it (Herrup 2015). Abeta comes in two main forms that differ in length by only two amino acids: amyloid-beta (1-40) (Abeta40) and amyloid-beta (1-42) (Abeta42). However, a recent study suggests many other Abeta fragments are also prevalent in AD brain (Wildburger et al. 2017). Studies suggest Abeta42 is more toxic than Abeta40 (Kuperstein et al. 2010). This may be because it more readily forms amyloid fibrils (Ogawa et al. 2011). Fibrils are the most prevalent form of Abeta found in those with AD, but oligomers appear to be the most toxic form (Sakono and Zako 2010). Abeta42 has been shown to stimulate inflammation (White et al. 2005) as well as form channels in cellular membranes that disrupt ion homeostasis in

neurons (Furukawa et al. 1994; Weiss et al. 1994). The amyloid plaques are typically extracellular and are thought to disrupt synaptic signaling (Ferreira et al. 2015).

Abeta appears to have intrinsically unstructured N- and C-termini with some secondary structure in the middle regions of the protein. Studies have found that the central hydrophobic cluster (CHC, residues 17-21) plays an important role in forming amyloid plaques (Wurth et al. 2002). Residues 24-26 and 31-34 appear to be important for fibrillization of Abeta40 (Williams et al. 2004). While it has been suggested that Abeta monomers in solution do not have secondary structure, there are studies that suggest Abeta does have secondary structure that is critical for amyloid formation (Chen and Glabe 2006). Although Abeta has been extensively studied, there is a lot of variability in experimental results possibly due to differences in experimental conditions. Therefore, examining amyloidogenic proteins for similarities using a novel computational approach could provide insights into structural features that other methods could not address.

Other Amyloidoses of the CNS. The brains of Parkinson's disease patients have cytoplasmic inclusions known as Lewy bodies (LBs) (Braak et al. 2003). LBs are mainly composed of α-syn (Tu et al. 1998), a 140 amino acid protein that forms alpha-helices when in a hydrophobic, membrane-like environment (Ulmer et al. 2005). While α-syn can form fibrils, it is thought that oligomers are the most toxic form of the protein (Conway et al. 2000). The α-syn protein has been shown to inhibit synaptic transmission through a variety of mechanisms (Stefanis 2012), including altering the distribution of SNARE proteins (Garcia-Reitböck et al. 2010) and decreasing the release of neurotransmitters (Nemani et al. 2010).

Human prion protein is an infectious protein that is the cause of TSEs. There are several types of TSEs that involve the conversion of the cellular form of the prion protein (PrP^{C}) to the infectious form (PrP^{SC}) (Colby and Prusiner 2011). The concept of infectious proteins was proposed by Prusiner in 1982 (Prusiner 1982). While hPrP often forms amyloids *in vivo* (Colby et al. 2007), amyloid formation is not required to cause disease (Wille et al. 2000). Like α-syn and Abeta, there is evidence that oligomeric PrP species are more toxic than fibrils for pathogenesis (Sokolowski et al. 2003). Because both α-syn and hPrP can form amyloids, they are included in the group of amyloidogenic proteins studied in Chapter 3.

Lysozyme Amyloidosis as a Model for General Amyloidosis. Lysozyme is a 14.7 kDa antimicrobial protein discovered by Alexander Fleming (Fleming 1922) and found in secretions such as tears (McDermott 2013) and saliva (van't Hof et al. 2014). While wild type (WT) lysozyme does not form amyloids *in vivo*, rare amyloidogenic mutants of lysozyme exist and are known to cause human disease (Granel et al. 2006). Because lysozyme is a well-studied protein and single amino acid substitutions can cause lysozyme to form amyloids, it is a useful model for studying the general process of amyloidogenesis (Merlini and Bellotti 2005). Several lysozyme point mutations are catalogued in the database Online Mendelian Inheritance in Man (OMIM 153450). These include I56T (Pepys et al. 1993), D67H (Pepys et al. 1993), W64R (Valleix et al. 2002), and F57I (Yazaki et al. 2003). It is generally thought that these mutations cause regions of instability between the alpha and beta domains, leading to unfolding (Booth et al. 1997). As the lysozyme molecules unfold, new intermolecular interactions can form between them, allowing

lysozyme to form amyloid plaques. While regions of instability have been clearly demonstrated to be involved (Booth et al. 1997), there may be other factors involved in amyloidogenesis. Applying residue interaction network analysis to lysozyme structures available in the Protein Data Bank (PDB) may yield new insights into the primary nucleation of lysozyme amyloids. Since lysozyme is a model for general amyloidogenesis, understanding the residue interaction network changes that occur in amyloidogenic variants of lysozyme could provide insight into the primary nucleation of other proteins as well. A better understanding of the amyloidogenic process could lead to more effective pharmacological interventions.

Residue Interaction Networks Provide a Novel Perspective into Protein Structure and Function

Complex networks analysis is a growing field that applies network science to a variety of systems (Estrada 2012). Proteins can be abstracted into a network of interacting amino acid residues where a node is represented by an amino acid and an edge represents a likely connection between amino acid residues (Di Paola et al. 2013; Yan et al. 2014). Conceptualizing a protein's structure as a network has proven to be a powerful technique that has provided several insights into protein structure (Dokholyan et al. 2002; Amitai et al. 2004; Brinda and Vishveshwara 2005; Liu and Hu 2011; Hu et al. 2014; Emerson and Louis 2015; Di Paola and Giuliani 2015). There are a variety of metrics available to describe a network (Junker and Schreiber 2008). These include global network measures such as average clustering coefficient, average shortest pathlength, average degree, and number of nodes and edges. There also exist local measures of a network such as clustering

coefficient, degree of a node, betweenness, and stress. Both global and local metrics have proven useful for protein network analysis. Network metrics applied to residue interaction networks (RINs) have been associated with features of proteins such as folding dynamics (Dokholyan et al. 2002) and active sites (Amitai et al. 2004). RINs are most useful for analyzing protein structure when combined with traditional 3D structural data and sequence data.

As described above, the 3D structures and sequences of monomeric and soluble amyloidogenic proteins are generally very well studied, but there have been no strong sequence or structural similarities found among them that suggest common features involved in amyloidogenesis (Knowles et al. 2014). Adding RIN data could better identify common features in amyloidogenic proteins. RINs provide a novel perspective of amyloidogenic proteins that could lead to the generation of information useful in further refining the current model of primary nucleation of amyloid fibrils.

As mentioned previously, Abeta plaques are a hallmark of AD. One of the hypotheses for the toxic effects of Abeta focuses on the potential of Abeta to cause oxidative damage (Smith et al. 1994; Smith et al. 1998; Rottkamp et al. 2001; Boyd-Kimball et al. 2005; Boyd-Kimball et al. 2006). Oxidative damage is increased in those with AD (Castegna et al. 2003; Perluigi et al. 2009) and in animal models of AD (Praticò et al. 2001). One of the enzymes especially sensitive to oxidative inactivation is glutamine synthetase (GLUL) (Butterfield et al. 1997), an enzyme that ligates ammonia and glutamate to produce glutamine. GLUL is oxidized *in vitro* after Abeta42 treatment (Boyd-Kimball et al. 2005) and has been shown to be oxidized in patients with mild cognitive impairment (MCI) (Butterfield et al. 2006) and in a mouse model of AD (Shen et al. 2015). Because of the sensitivity of GLUL to

oxidative inactivation and the potential effects on ammonia metabolism due to oxidative damage, I expanded my research to examine the role of ammonia metabolism enzymes on blood ammonia levels and the potential neurotoxicity when enzyme activity is altered in disease conditions.

Specific Aim 2

To create a mathematical model to determine if the amount of dietary protein and degree of liver function interact to affect blood ammonia levels and to determine if the predicted changes could affect neuron health. This aim addresses hypothesis **(B)** and investigates the potential role of ammonia metabolism in neural function using an organismal and tissue level approach.

Urea Cycle, CPS1, GLS, and GLUL in Ammonia Metabolism

Ammonia is a toxic byproduct of amino acid catabolism (Auron and Brophy 2012) that is processed in mammals into the relatively nontoxic molecule urea by enzymes of the urea cycle. The urea cycle is composed of a series of enzymes located mainly in the liver, but also in the kidneys and small intestine. The first committed step of the urea cycle is catalyzed by the enzyme carbamoyl phosphate synthetase 1 (CPS1), a mitochondrial protein that synthesizes carbamoyl phosphate from ammonia, carbonic acid, and ATP. CPS1 deficiency, a disease caused by mutations in the *CPS1* gene that reduce the enzyme's activity, results in increased systemic ammonia levels (Klaus et al. 2009). Severe CPS1 deficiency typically leads to death at an early age due to the sensitivity of the brain to high ammonia levels.

Many other enzymes exist that are involved in ammonia metabolism but are not part of the urea cycle. These include glutamine synthetase (GLUL), as mentioned earlier, and glutaminase (GLS). Glutamine is relatively inert as a signaling molecule (Albrecht et al. 2010). However, glutamine can be broken down into ammonia and glutamate by the enzyme glutaminase (Albrecht and Norenberg 2006). Glutamate is an important neurotransmitter involved in synaptic function (Zhou and Danbolt 2014) and is discussed in more detail below. GLUL has a very low K_m (high affinity for substrate) (Listrom et al. 1997), so it efficiently processes free ammonia into glutamine when glutamate is present. The interplay among these ammonia metabolism enzymes has important implications for a variety of diseases.

Liver Physiology and Hepatic Encephalopathy

The liver carries out a variety of critical physiological functions. One of the most important functions is the removal of ammonia from the blood to create urea for excretion. This is carried out through a series of enzymes that are spatially separated in the acinus, the functional unit of the liver. Briefly, the hepatic portal vein carries blood from the small intestine to the liver. The blood then diffuses across about 22 hepatocyte cell layers in the acinus and then collects in a branch of the central vein. The hepatocytes of the acinus are divided into three zones which each express a set of enzymes to metabolically process the compounds in the blood coming from the small intestine (Häussinger et al. 1992; Brosnan and Brosnan 2009; Lautt 2009). Zone 1 is closest to the hepatic portal vein. These hepatocytes express the enzymes CPS1 (Moorman et al. 1989) and GLS (Moorman et al. 1994). Zone 2 largely contains the same ammonia metabolism enzymes as zone 1 but with

lower levels of GLS (Moorman et al. 1989; Moorman et al. 1994). Zone 3, however, is closest to the central vein and contains GLUL instead of CPS1 and GLS (Gebhardt and Mecke 1983). The separation of these enzymes makes ammonia metabolism more efficient.

Liver damage causes changes in the metabolic functions of the liver (Gebhardt and Reichen 1994; Fleming and Wanless 2013). Hepatic encephalopathy is a condition where liver damage leads to an accumulation of potentially neurotoxic factors in the blood, impairing cognitive function (Butterworth 2015). Liver cirrhosis has been shown to cause changes in the activities of CPS1 and GLUL (Gebhardt and Reichen 1994; Fleming and Wanless 2013). When these enzyme activities are altered, the rate at which ammonia metabolism occurs is altered as well, leading to higher levels of ammonia in the blood of patients with liver damage (Butterworth 2003). The higher levels of blood ammonia in those with hepatic encephalopathy cause neural dysfunction, leading to the clinical symptoms of disease.

Effects of Blood Ammonia on Neural Cells

Most ammonia (NH_3) becomes protonated at physiological pH and is present in the body as the ammonium ion (NH_4^+). For simplicity, "ammonia" is used in this dissertation to include both chemical species unless otherwise noted. Uncharged ammonia can passively cross the blood-brain barrier (BBB), while the charged ammonium ion cannot (Auron and Brophy 2012). Because the brain has a lower pH than blood, once ammonia passes through the BBB and becomes protonated, it does not pass back through so easily, leading to a higher concentration of ammonium ions in the brain compared to the blood (Auron and Brophy 2012). Once in the brain, ammonia can cause damage to neural cells and interrupt

cognitive function in a variety of ways. First, ammonia can directly exert toxicity on neurons and glia. Studies have demonstrated that increased ammonia levels cause increased reactive oxygen and nitrogen species production (Bobermin et al. 2015), leading to oxidative damage. Furthermore, ammonium ions compete with potassium for transport into astrocytes and neurons, leading to problems with inhibitory neurotransmission in the cortex (Rangroo Thrane et al. 2013). Next, high ammonia levels disrupt the glutamate-glutamine balance in the brain (Braissant et al. 2013). Glutamate is an excitatory neurotransmitter involved in learning and memory (Esposito et al. 2013). Disruption of glutamate metabolism and signaling by high ammonia levels could lead to some of the symptoms of neurological diseases. Either too much or too little glutamate signaling has been shown to have negative effects on the brain (Myhrer 1998). The results of Specific Aim 2 add further evidence that increased ammonia levels resulting from pathophysiologically relevant enzyme activity changes are directly toxic to neurons. Because of these results and the established role of glutamate signaling in neuronal function and AD, I investigated a potential connection between ammonia metabolism genes and three neurological and metabolic diseases: AD, major depressive disorder (MDD), and type-2 diabetes (T2D).

Specific Aim 3

To determine if there is an association between single nucleotide polymorphisms (SNPs) in the *GLS*, *CPS1*, and *GLUL* genes and AD, MDD, and T2D, and to predict the effects of any associated SNPs in the disease processes. This aim addresses hypothesis **(B)** where I

suggest that altered ammonia metabolism stimulates neuropathology on a molecular and cellular level.

Ammonia Metabolism Could Play a Common Role in AD, T2D, and MDD

Proper organismal ammonia metabolism is critical for normal physiology, but it is especially important for brain function. Because of its very high toxicity and its production by a central metabolic pathway, ammonia metabolism may be altered in a variety of diseases where central metabolism is altered. Therefore, we hypothesized that ammonia and glutamate metabolism may play a role in the pathogenesis of AD, MDD, and T2D. While these diseases have many differences, cortical thinning has been associated with all three diseases (Du et al. 2007; Tu et al. 2012; Yoon et al. 2017). Memantine is one of the handful of drugs available for the treatment of AD. It is an NMDA receptor antagonist, so it reduces glutamate excitotoxicity in neurons. Overactivation of NMDA receptors results in the excessive opening of plasma membrane calcium channels, resulting in calcium-induced cell death. Memantine has been shown to slightly slow the progression of AD, reinforcing that glutamate signaling is important in AD pathogenesis. However, memantine administration has also been shown to have positive effects in patients with MDD (Amidfar et al. 2017) and in a mouse model of T2D (Iwanami et al. 2014; Huang et al. 2017). Since memantine may be effective against all three of these diseases, there are likely common features of pathogenesis among them. Exploring common disease features may lead to better treatments for patients with a variety of diseases. This suggests that drugs targeting ammonia and glutamate metabolism and signaling may be worthwhile pharmacological interventions.

In addition to the link between AD, MDD, and T2D and glutamate and ammonia metabolism and signaling, the comorbidity of the three diseases suggests a possible link among them. For example, T2D nearly doubles the risk of developing MDD (Anderson et al. 2001; Ali et al. 2006), and those with a history of depression have an increased risk of developing AD (Geerlings et al. 2008). Furthermore, a strong link between T2D and AD appears to be established (Ott et al. 1999; Luchsinger et al. 2005; Gudala et al. 2013; Li et al. 2015). Ammonia metabolism and its role in glutamate signaling may partially explain the comorbidity, the benefit of NMDA receptor antagonists, and the similar tissue pathology of these diseases with neurological components. Furthermore, since each of these diseases has a heritable component (Gatz et al. 1997; Sullivan et al. 2000; Willemsen et al. 2015), investigating genetic abnormalities in ammonia metabolism genes in individuals with these diseases may further support a causative role for dysfunctional ammonia and glutamate metabolism in the onset and progression of the disorders.

Amino Acid and Ammonia Metabolism in AD. Since 1992, Abeta has been a major focus of AD research (Hardy and Higgins 1992). However, Abeta is likely not the only factor involved in AD pathogenesis. The year after the amyloid hypothesis was proposed, Seiler proposed the ammonia hypothesis of AD (Seiler 1993). However, due to the popularity of the amyloid hypothesis, it has received little attention although the data that support it are strong. In light of the numerous failed AD drug trials that sought to prevent Abeta fibrilization or remove fibrils that were already present, many researchers have suggested that the investigation of other hypotheses of AD pathogenesis is warranted (Herrup 2015). Of the drugs currently approved to treat AD, none of them targets Abeta, but memantine

alters glutamate signaling. Therefore, strategies targeting ammonia and glutamate metabolism may lead to more effective treatments. Seiler has reviewed the body of evidence that supports the role of ammonia in AD (Seiler 2002). Briefly, subjects with AD tend to have high levels of ammonia in their plasma (Fisman et al. 1985), cerebrospinal fluid (CSF), and autopsied brains than age-matched controls (Hoyer et al. 1990). The cognitive symptoms of those with CPS1 deficiency or hepatic encephalopathy (although not identical with AD) demonstrate the effects of hyperammonemia on the CNS (Raabe 1987). Together, this evidence warrants further research.

In addition to increased ammonia levels in AD patients, amino acid level changes in those with AD have also been thoroughly investigated. Several amino acids have altered concentrations in blood, CSF, or autopsied brain tissue of AD patients (Chapter 5). Because amino acids release ammonia when catabolized and are precursors for the synthesis of a variety of metabolites, their altered levels in AD patients could lead to a variety of disease symptoms. The changes in amino acid levels in AD patients and animal models are thoroughly reviewed in Chapter 5.

Ammonia Metabolism in MDD and T2D. Ammonia and glutamate metabolism have also been linked to MDD and T2D. In addition to the positive effects of memantine on the symptoms of MDD patients and in a mouse model of T2D, some studies have reported associations between ammonia metabolism genes and MDD and T2D. Changes in the activity of *GLS* in a diabetes rat model were found (Ardawi 1987), and it is also known that glutamate signaling pathways are altered in those with MDD (Bernard et al. 2011).

Overview of the Dissertation

In the first part of this dissertation, I discuss the results of studies seeking to find similarities among amyloidogenic proteins by combining 3D structural analysis with RIN analysis. Amyloidogenic lysozyme was used as a model of an amyloidogenic protein to demonstrate the usefulness of combining 3D structural data and RIN data in the study of primary nucleation (Chapter 2). Next, a group of amyloidogenic proteins was compared to random network controls and a group of real proteins to detect similarities among the amyloidogenic proteins (Chapter 3). Then, Abeta, as an example from a prevalent disease, was explored in more detail to find novel residue interactions that may be involved in primary nucleation of amyloid fibrils (Chapter 3).

In the second part of this dissertation, the results of the studies aimed at investigating the potential role of ammonia in a variety of neurological diseases are discussed. First, a computational model was built to investigate the interactions among the activities of enzymes involved in ammonia metabolism, dietary protein intake, and blood ammonia levels and their potential role in hepatic encephalopathy (Chapter 4). Next, the literature on amino acid and ammonia metabolism changes that occur in AD was thoroughly reviewed (Chapter 5). Finally, single nucleotide polymorphisms (SNPs) in ammonia metabolism genes were found to be linked to AD, MDD, and T2D, suggesting the potential for altered ammonia metabolism to play an important role in a variety of diseases with neurological symptoms (Chapter 6). By combining the study of amyloid forming proteins such as Abeta in Alzheimer's disease and ammonia metabolism in hepatic encephalopathy, AD, MDD, and T2D, a more complete understanding of diseases with neurological and metabolic components has been obtained.

CHAPTER 2

IN SILICO PREDICTION OF NOVEL RESIDUES INVOLVED IN AMYLOID PRIMARY NUCLEATION OF HUMAN I56T AND D67H LYSOZYME

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Abstract

Amyloidogenic proteins are most often associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, but there are more than two dozen human proteins known to form amyloid fibrils associated with disease. Lysozyme is an antimicrobial protein that is used as a general model to study amyloid fibril formation. Studies aimed at elucidating the process of amyloid formation of lysozyme tend to focus on partial unfolding of the native state due to the relative instability of mutant amyloidogenic variants. While this is well supported, the data presented here suggest the native structure of the variants may also play a role in primary nucleation. Three-dimensional structural analysis identified lysozyme residues 21, 62, 104, and 122 as displaced in both amyloidogenic variants compared to wild type lysozyme. Residue interaction network (RIN) analysis found greater clustering of residues 112-117 in amyloidogenic variants of lysozyme compared to wild type. An analysis of the most energetically favored predicted dimers and trimers provided further evidence for a role for residues 21, 62, 104, 122, and 112-117 in amyloid formation. This study used lysozyme as a model to demonstrate the utility of combining 3D structural analysis with RIN analysis for studying the general process of amyloidogenesis. Results indicated that binding of two or more amyloidogenic lysozyme mutants may be involved in amyloid nucleation by placing key residues (21, 62, 104, 122, and 112-117) in proximity before partial unfolding occurs. Identifying residues in the native state that may be involved in amyloid formation could provide novel drug targets to prevent a range of amyloidoses.

Keywords

Amyloidosis, Lysozyme, Residues Interaction Networks, Native Structure, *B*-Factor

Background

Amyloidoses are a group of diseases defined by the formation of protein aggregates characterized by stacks of cross-beta sheets (Greenwald and Riek 2010). There are dozens of different amyloid diseases caused by a variety of both wild type (WT) and mutant forms of proteins (Knowles et al. 2014). Some of the most well-known amyloidoses are neurodegenerative diseases such as Alzheimer's disease (involving amyloid-beta peptide) and Parkinson's disease (involving alpha-synuclein protein). However, not all amyloid diseases affect the brain. Lysozyme amyloidosis is a rare disease characterized by the deposition of amyloid fibrils of the enzyme lysozyme. Lysozyme was discovered by Alexander Fleming in 1922 (Fleming 1922) and is an antimicrobial enzyme synthesized by hepatocytes, cells of the gastrointestinal system, and macrophages (Granel et al. 2006). Lysozyme amyloidosis has no known effective treatment and leads to lysozyme amyloid deposits typically concentrated in the liver (Harrison et al. 1996), spleen, gastrointestinal tract (Granel et al. 2002), and kidneys (Gillmore et al. 1999). Lysozyme amyloidosis is thought to be largely caused by subtle structural changes of the protein caused by genetic mutations that lead to pockets of local instability and a greater likelihood of partial unfolding (Canet et al. 1999). The Online Mendelian Inheritance in Man (OMIM) database (Amberger et al. 2015) entry for lysozyme (OMIM ID 153450) reports four lysozyme variants that are associated with the disease: I56T (Pepys et al. 1993), D67H (Pepys et al. 1993), W64R (Valleix et al. 2002), and F57I (Yazaki et al. 2003).

Lysozyme has long been used as a model for studying protein structure and folding. Since lysozyme is structurally and functionally well-characterized, the protein provides a useful model for understanding the complex process of amyloid fibril formation (Merlini and Bellotti 2005). Several studies have investigated the role of amyloidogenic mutations on lysozyme amyloid formation with a focus on the first identified mutations, I56T and D67H. Studies that examined the crystal structure of the WT and I56T variant suggest very little difference in the native structure of these enzymes (Funahashi et al. 1996). The D67H variant, however, destroys the hydrogen bonds that stabilize the beta domain, leading to the displacement of a long loop of residues (Booth et al. 1997). Because the obvious loop displacement between the WT and D67H mutant is not present in the I56T mutant, it is thought that this change is not responsible for amyloidogenesis. Instability of the I56T variant may be caused by subtle changes in bonding between alpha and beta domains of lysozyme; similar bonding changes are also evident in the D67H variant (Booth et al. 1997).

Since the structure of proteins in amyloid plaques are different from the native structure, amyloidogenic proteins must at least partially unfold during amyloidogenesis. Most studies focus on the unfolding process of lysozyme instead of differences in the native structure. The amyloidogenic proteins likely spend more time partially unfolded, providing more opportunities for unfolded segments to interact and aggregate in the form of amyloid plaques (Canet et al. 1999). The importance of the partially unfolded state for lysozyme amyloidosis has been demonstrated *in vitro* with the use of antibodies that stabilize the protein (Dumoulin et al. 2003; Chan et al. 2008). Studies have also shown that both the I56T and D67H mutants are less stable than WT lysozyme when heated (Booth et al. 1997), and the I56T mutant is also less stable than WT at low pH (Funahashi et al. 1996; Booth et

al. 1997; Morozova-Roche et al. 2000; Buell et al. 2011), further supporting a role for instability. However, other factors besides regions of protein instability may be involved in amyloidogenesis. Further studies that examine primary nucleation from different perspectives could provide more insight into this important process that is associated with a variety of diseases.

Residue interaction networks (RIN) abstract protein structure into a network of likely side-chain interactions with residues represented as nodes and interactions represented as edges, the connections between the nodes (Di Paola et al. 2013). Several metrics are available for studying networks and identifying subnetworks of interest (Junker and Schreiber 2008; Estrada 2012; Scardoni and Laudanna 2012). Many network features have been associated with and applied to protein structural and functional characteristics (Liu and Hu 2011; Scardoni and Laudanna 2012; Hu et al. 2014; Emerson and Louis 2015), demonstrating the relevance of RINs to structural biology. Clusters are particularly interesting in RIN analysis because they identify areas with many chemical interactions, suggesting structural rigidity or functional importance (Vishveshwara et al. 2002). RIN analysis is most useful when combined with 3D structural analysis (Amitai et al. 2004). This study uses the two-pronged approach of combining 3D structural analysis with RIN analysis to identify residues in the native structure that are likely involved in amyloid formation.

Methods

Three-Dimensional Structure Visualization and Structure Comparison

The Online Mendelian Inheritance in Man (OMIM) database (Amberger et al. 2015) was searched for mutations in lysozyme that have been associated with amyloidosis. The Protein Data Bank (PDB) (Berman et al. 2000) was then searched for human lysozyme structures with these mutations, resulting in a dataset of WT human lysozyme (PDB ID: 1REX, (Muraki et al. 1996)) and two amyloidogenic variants, I56T (PDB ID: 1LOZ, (Booth et al. 1997)) and D67H (PDB ID: 1LYY, (Booth et al. 1997)). The 3D protein structure coordinates were downloaded from the PDB and visualized using UCSF Chimera v1.11.2 software (Pettersen et al. 2004). The 3D structures were overlapped using the MatchMaker application (Meng et al. 2006) in UCSF Chimera with default settings, and the root mean square deviations (RMSDs) for the full residues from the wild type (1REX) structure of lysozyme were calculated in the Multialign Viewer (Meng et al. 2006). Side chains that had a different location when compared to WT lysozyme in both amyloidogenic variants were selected for further study. Because the resolution of the PDB files used for the comparison was less than or equal to 1.8 angstroms, only residues with a RMSD from WT greater than or equal to 1.9 angstroms were considered.

Generating Residue Interaction Networks and Calculating Clusters and Metrics

To detect network clusters in the proteins, the PDB files were converted to GML format using the Protein Graph Converter software from the Protein Graph Repository (PGR) (Dhifli and Diallo 2016). Each alpha carbon was considered a node, and an edge was drawn between every alpha carbon within seven angstroms of another. The GML files were

then analyzed for clusters using the MCODE application in the network analysis software Cytoscape v3.4.0 (Shannon et al. 2003). Only clusters with MCODE scores greater than or equal to 5.00 were selected for further analysis. The residues involved in the clusters in the amyloidogenic mutants were compared to those identified in WT lysozyme. As with the 3D structure comparison, cluster changes that are in common between the amyloidogenic mutants and different from WT lysozyme were selected for further analysis. UCSF Chimera was used to calculate the average residue *B*-factor for each of the clusters. The Pearson correlation of MCODE scores for the selected clusters of each of the lysozyme PDB files with the average *B*-factor for the clusters was calculated using GraphPad Prism 7, and a p-value of < 0.05 was considered significant.

Generating Predicted Dimer and Trimer Structures and Calculating Interprotein Bond Number and Energies

ClusPro v2.0 software (Kozakov et al. 2017) was used to generate predicted structures of homodimers, homotrimers, and heterodimers for WT lysozyme and the amyloidogenic variants. After multimer generation, the resulting PDB files for the top predicted dimer and trimer structures were edited so that each lysozyme protein was given a unique name. Next, the edited PDB files were uploaded to the Residue Interaction Network Generator (RING) v2.0 software (Piovesan et al. 2016), and residue interaction networks were created using a strict distance threshold between the closest atoms of residues separated by at least two other residues. Multiple edges per residue pair were allowed but only one edge per interaction type. The resulting graph files were analyzed for the number of interactions and overall bonding energy occurring between lysozyme

proteins in dimers or trimers. The number and bond energies of the interprotein interactions were analyzed for the whole complexes and the residues of interest to provide information about the relative importance of the residues of interest to the formation of dimers and trimers.

Results

Three-Dimensional Structural Comparison of Lysozyme

Three-dimensional structure overlaps from the MatchMaker software revealed residues of the amyloidogenic variants that differed from WT lysozyme. The D67H variant diverges more from WT lysozyme than the I56T variant. However, there are only four residues with a RMSD greater than or equal to 1.9 angstroms that were shared by both the I56T and the D67H amyloidogenic lysozyme variants: residues 21, 62, 104 and 122. In all four cases, the structural changes are in proximity to each other in the 3D structure (**Figure 2.1**).

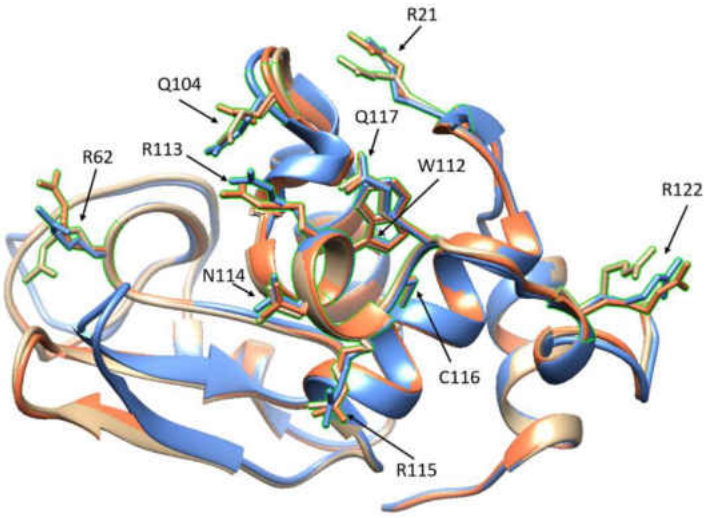

Figure 2.1: Three-dimensional overlap of lysozyme structures 1REX (WT, tan), 1LOZ (I56T, orange), and 1LYY (D67H, blue). The side chains of residues 21, 62, 104, 112-117, and 122 are shown and outlined in green.

Residue Interaction Network Clustering Analysis of Lysozyme Structures

The 3D structure of lysozyme and its resulting PGR residue interaction network (RIN) representation are shown in **Figure 2.2**. The MCODE application in Cytoscape revealed four clusters in each of the lysozyme structures that had an MCODE score of greater than 5.00. Some of the clusters in each of the lysozyme variants involved similar or identical sets of residues. As before, we focused on the differences from the WT clusters that were present in both amyloidogenic proteins. The most robust and consistent difference was the cluster around residues 112-117. The MCODE score for the cluster containing these residues in WT lysozyme was 5.11, but it increased in both amyloidogenic variants to 6.00. Residues 104 and 106-108 are clustered with 112-117 in WT lysozyme but not in I56T and D67H. The average *B*-factors of the clusters containing residues 112-117 also decreased in both amyloidogenic variants compared to WT lysozyme. These results are shown in **Table 2.1**. Residues 112-117 are shown in Figure 2.1. There was a statistically significant negative correlation ($r^2 = 0.44$, $p = 0.0184$) between MCODE scores for the top four clusters in each of the three lysozyme network structures and the average *B*-factor for each cluster. The residues are in proximity to the other residues of interest as shown in Figure 2.1.

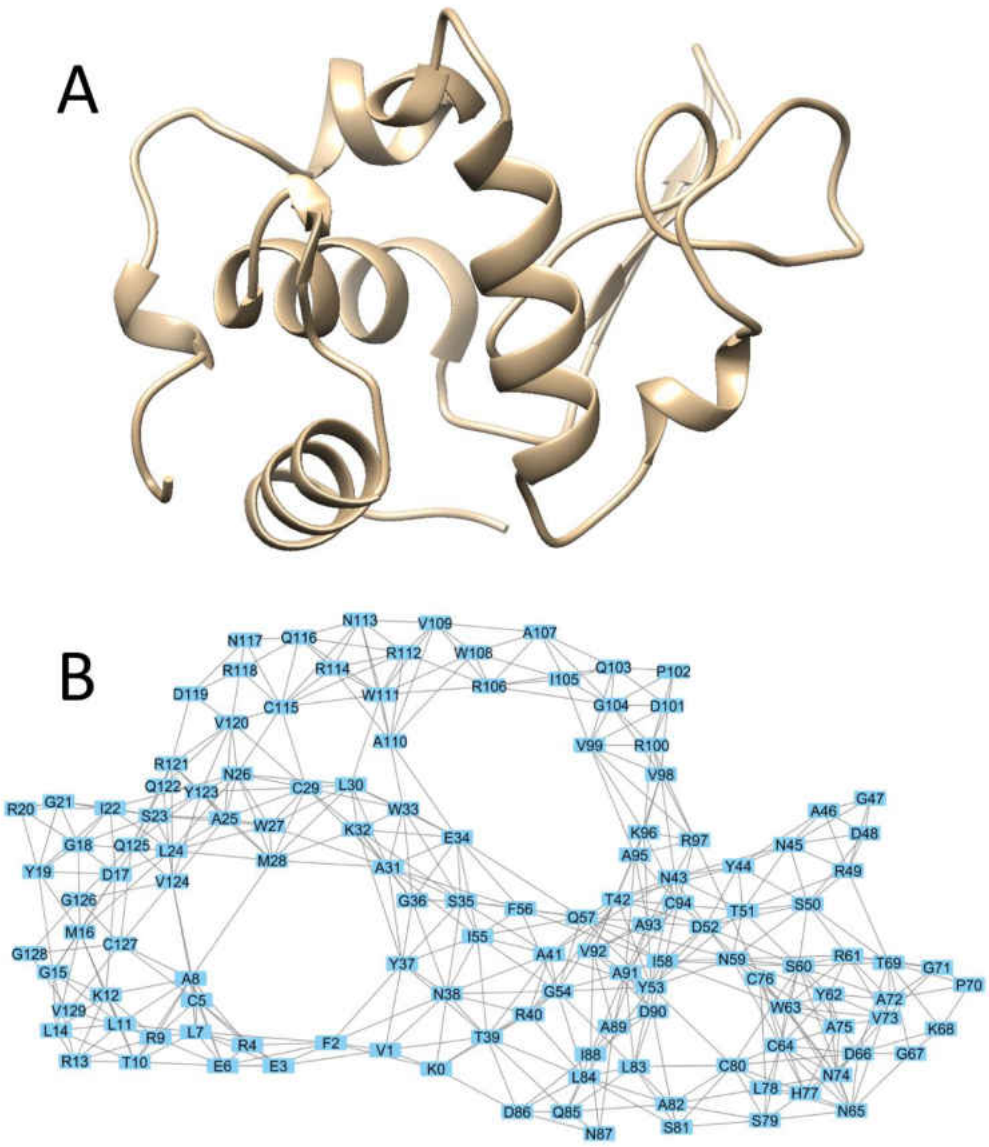

Figure 2.2: Three-dimensional and network structures of lysozyme. A) The three-dimensional structure of WT lysozyme, PDB 1REX. B) Network representation of WT lysozyme with amino acids represented as nodes and edges drawn between alpha carbons within seven angstroms of each other. Residue numbering starts at zero only for this image.

Table 2.1: RIN clusters in WT and amyloidogenic variants of lysozyme

Lysozyme Structure	Cluster Rank	MCODE Score	Residues Involved	Average Residue B-Factor
WT				14.88
	1	6.36	92-103	12.35
	2	6.00	32-37	11.71
	3	5.60	7-13, 19, 23-29, 31	9.72
	4	5.11	104, 106-108, 112-117	19.09
I56T				16.06
	1	6.00	112-117	17.53
	2	6.00	92-98	10.54
	3	5.60	7-13, 19, 23-29, 31	10.39
	4	5.00	122-126	29.15
D67H				13.24
	1	6.50	92-100	7.83
	2	6.00	112-117	14.99
	3	6.00	32-37	8.21
	4	5.75	7-13, 17-19, 23, 25-29, 31	8.12

Interprotein Bonds Involving Residues 21, 62, 104, 112-117, and 122 in Predicted Lysozyme Dimers and Trimers

The top-rated ClusPro models of dimer and trimer structures for each of the variants of lysozyme are shown in **Figure 2.3** (homodimers), **Figure 2.4** (homotrimers), and **Figure 2.5** (heterodimers). The number of interprotein residue interactions and the strength of the bonding energy for these interactions for all residues and for the residues of interest were quantified using RING 2.0 software, and the results are shown in **Table 2.2** (homodimers), **Table 2.3** (homotrimers), and **Table 2.4** (heterodimers).

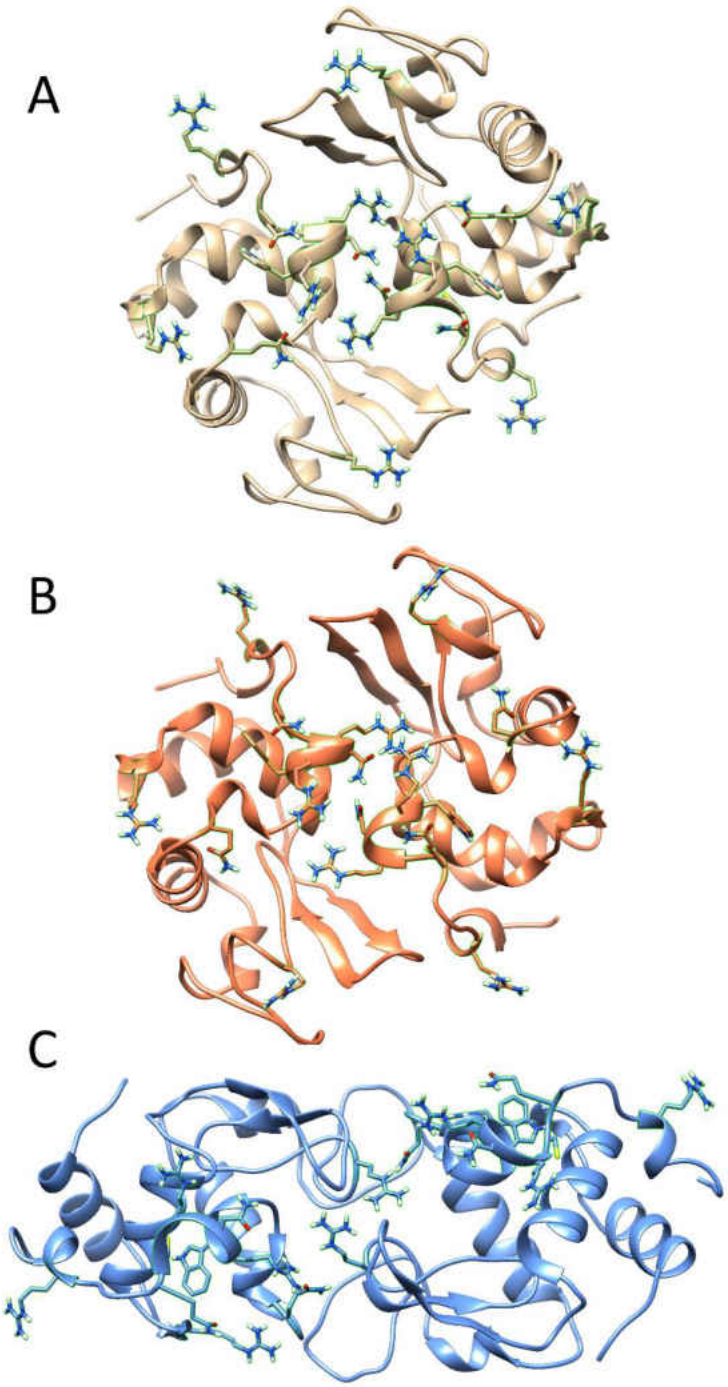

Figure 2.3: Predicted 3D structures of WT or mutant lysozyme homodimers. A) WT: WT, B) I56T: I56T, and C) D67H: D67H. The side chains of residues 21, 62, 104, 112-117, and 122 are shown and outlined in green.

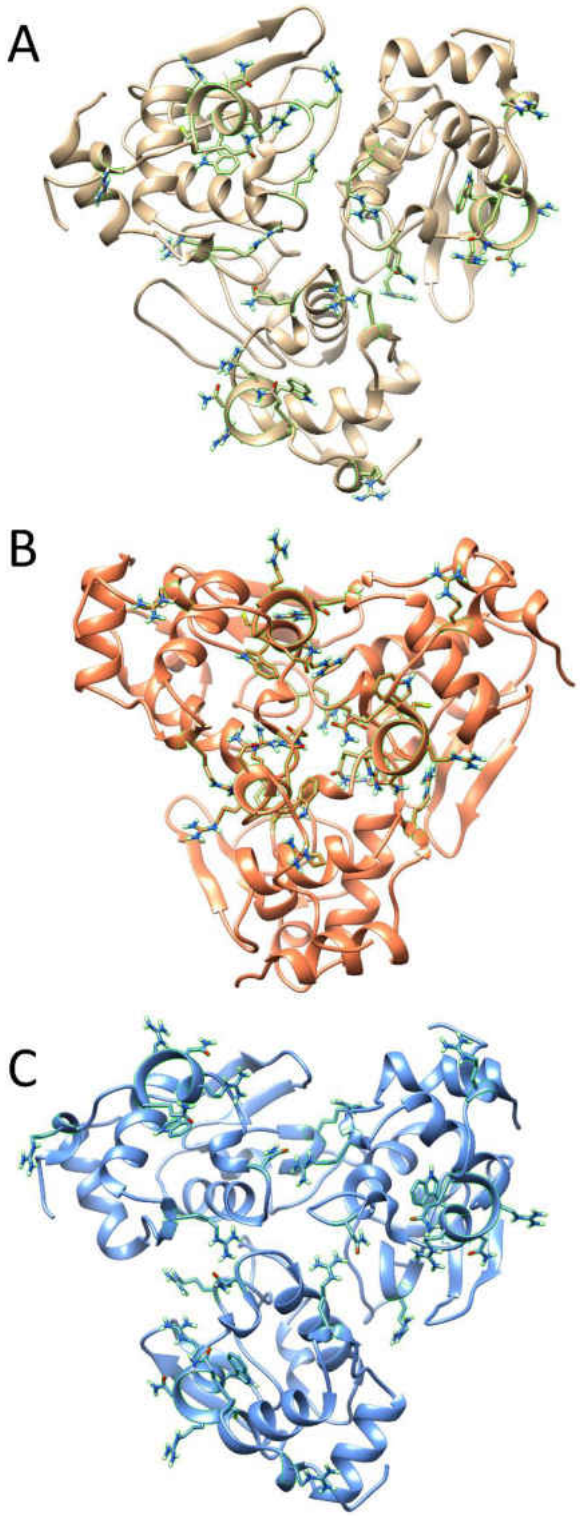

Figure 2.4: Predicted 3D structures of WT or mutant lysozyme homotrimers. A) WT: WT: WT, B) I56T: I56T: I56T, and C) D67H: D67H: D67H. The side chains of residues 21, 62, 104, 112-117, and 122 are shown and outlined in green.

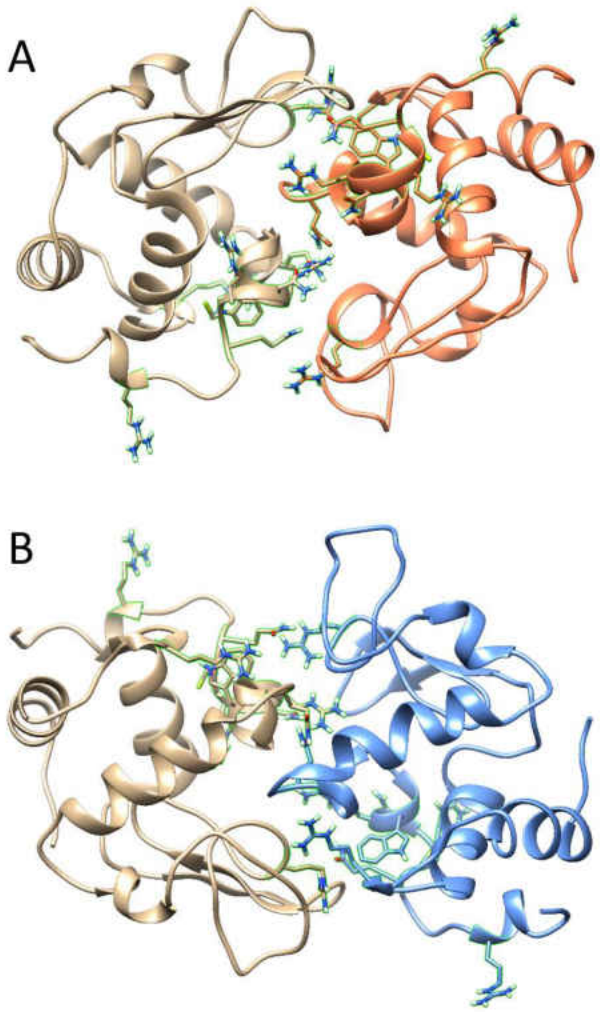

Figure 2.5: Predicted 3D structures of heterodimers of each of the amyloidogenic lysozyme variants with WT lysozyme. A) WT: I56T, B) WT: D67H. The side chains of residues 21, 62, 104, 112-117, and 122 are shown and outlined in green.

Table 2.2 ClusPro predicted interprotein binding energies for WT and mutant lysozyme homodimers

Lysozyme Structure	Number of Interprotein Residue Interactions		Interprotein Bond Energy (kJ/mol)	
	Total	Residues of Interest (% of Total)	Total	Residues of Interest (% of Total)
WT	100	36 (36 %)	1019	398 (39.1 %)
I56T	71	35 (49.3 %)	768	442 (57.6 %)
D67H	61	18 (29.5 %)	594.6	246 (41.4 %)

Table 2.3 ClusPro predicted interprotein binding energies for WT and mutant lysozyme homotrimers

Lysozyme Structure	Number of Interprotein Residue Interactions		Interprotein Bond Energy (kJ/mol)	
	Total	Residues of Interest (% of Total)	Total	Residues of Interest (% of Total)
WT	89	2 (2.2 %)	824.8	12 (1.5 %)
I56T	133	74 (55.6 %)	1392.8	817 (58.7 %)
D67H	108	24 (22.2 %)	1060.8	320 (30.2 %)

Table 2.4 ClusPro predicted interprotein binding energies for WT and mutant lysozyme heterodimers

Lysozyme Structures	Number of Interprotein Residue Interactions		Interprotein Bond Energy (kJ/mol)	
	Total	Residues of Interest (% of Total)	Total	Residues of Interest (% of Total)
WT: I56T	48	25 (52.1 %)	481	238 (49.5 %)
WT: D67H	49	27 (55.1 %)	482.6	289.6 (60.0 %)

For homodimers, residues of interest made up 36% of the number of interprotein residue interactions in WT lysozyme, contributing to 39.1% of the predicted interprotein bonding energy. The overall number of interprotein residue interactions increased to 49.3% of the total in the I56T variant, but it decreased to 29.5% of the total number in the D67H variant. However, the total percentage of interprotein bonding energy contributed by the residues of interest increased for both amyloidogenic variants even though the total interprotein bonding energy for dimers was less than WT dimers.

Trimers showed different trends from the homodimers (Table 2.3). The total number of interprotein residue interactions increased for I56T (133 interactions) and D67H (108 interactions) compared to WT (89 interactions). The contribution of the residues of interest to the total number of interprotein residue interactions increased from 2.2% for the WT to 55.6% for the I56T variant and 22.2% for the D67H variant. Interprotein bonding energy showed similar trends. The total interprotein bonding energy increased from 824.8 kJ/mol in the WT to 1392.8 kJ/mol in the I56T variant and 1060.8 kJ/mol in the D67H variant. Residues of interest contributed 1.5% of the total interprotein bonding energy in the WT but 58.7% in the I56T variant and 30.2% in the D67H variant.

Heterodimers (WT: I56T and WT: D67H) showed fewer and less energetic interprotein interactions than homodimers (Table 2.4). The WT: I56T variant heterodimer had 48 interprotein interactions and 481 kJ/mol interprotein bonding energy, and the WT: D67H variant heterodimer had 49 interprotein interactions and 482.6 kJ/mol bonding energy. In both cases, there was a high reliance on the residues of interest for the bonding. The residues of interest made up more than 50% of the number of interprotein bonding interactions and nearly 50% of the interprotein bonding energies of both heterodimers.

Discussion

Three-Dimensional Structural Comparison Suggests Residues 21, 62, 104, and 122 May Be Involved in Lysozyme Amyloidogenesis

Comparing 3D protein structures revealed that both amyloidogenic variants of lysozyme analyzed differ from WT lysozyme in the location of residues 21, 62, 104, and 122. Following the reasoning of Booth and colleagues (Booth et al. 1997), because these differences are common to both amyloidogenic variants, they suggest these residues may play a role in the formation of amyloid fibrils. The four residues (21, 62, 104, and 122) are in proximity to each other in all three variants (Figure 2.1). As discussed above, the prevailing hypothesis for lysozyme amyloidosis is that the mutations disrupt the hydrogen bonds near the residues between alpha and beta domains, leading to partial unfolding followed by fibril formation (Canet et al. 1999; Johnson et al. 2005; Buell et al. 2011); interprotein interactions between the native structures are not thought to play a large role in unfolding. However, the consistency of the structural changes observed in both amyloidogenic variants compared to WT hints at a role for these residues in amyloidosis. We hypothesized that these residues may facilitate an interprotein interaction between native state amyloidogenic lysozyme proteins, contributing to the first steps of amyloidosis. To gather further support for this hypothesis, we examined the structures for network cluster changes.

Residues 112-117 May Also Be Involved in Lysozyme Amyloidogenesis

In addition to the 3D structural changes described above, amyloidogenic variants of lysozyme were associated with changes in network clusters. The network cluster

consisting of residues 112-117 in the I56T and the D67H variants had the greatest and most consistent cluster changes, showing an increased MCODE score in both variants compared to WT (Table 2.1). Residues 104 and 106-108 were included in the cluster containing residues 112-117 in WT lysozyme only, so the loss of this part of the cluster may also be important for the structural changes observed. More clustering has been shown to be associated with greater structural stability (Kannan and Vishveshwara 2000; Vishveshwara et al. 2002), so we hypothesized that residues 112-117 have greater structural stability in the amyloidogenic variants compared to WT lysozyme. To test this hypothesis, we used UCSF Chimera to calculate the average *B*-factor for each of the residues in each of the PDB files. The *B*-factor is a measure of flexibility where a lower *B*-factor indicates greater stability (Yuan et al. 2005). Even in our relatively small data set of three lysozyme structures, we found a statistically significant negative correlation ($r^2 = 0.44$, $p = 0.0184$) between MCODE scores and average *B*-factors for the top four clusters of each of the PDB files shown in Table 2.1. Consistent with the hypothesis of greater cluster stability, residues 112-117 had smaller average *B*-factors in amyloidogenic variants compared to the cluster containing these residues in WT lysozyme. Because most studies focus on the instability caused by amyloidogenic mutations, disrupting this cluster while stabilizing other regions may provide a novel therapeutic approach. The side chains of residues 112-117 are shown in Figure 2.1 along with the other residues of interest (21, 62, 104, and 122) from the 3D structural comparison. Residues 112-117 are in proximity to the residues identified through the 3D structural comparison. Therefore, residues 112-117 may also be involved in facilitating interactions between different lysozyme molecules and possibly

contribute to the primary nucleation of amyloid fibrils. To test this hypothesis, we simulated intermolecular interactions between native state structures.

Predicted Dimer and Trimer Structures Provide Further Evidence for the Involvement of Residues 21, 62, 104, 112-117, and 122 in Lysozyme Amyloidogenesis

To test the hypothesis that residues 21, 62, 104, 112-117, and 122 in the structure of amyloidogenic lysozyme variants are involved in primary nucleation, we used ClusPro docking software to predict the 3D structure of dimers and trimers of lysozyme for the PDB files. While ClusPro generates the structures of many predicted dimers and trimers, we only analyzed the top-ranked structures. Visual inspection of the predicted homodimers (Figure 2.3) suggested some of the residues of interest may be involved in dimer interactions. To test this hypothesis, we examined the number and strength of interprotein residue interactions (Table 2.2). The number of interprotein bonds was less for amyloidogenic variant homodimers, and there was no consistent trend with the percent contribution of the residues of interest to the number of interprotein bonds in the dimers. Both amyloidogenic variant homodimers had overall less bonding energy. However, the residues of interest contributed to a greater degree to the interprotein bonding energy compared to WT lysozyme. Overall, the contribution of the residues of interest to lysozyme homodimer formation in amyloidogenic variants was not as convincing as the evidence for their role in simulated trimer formation.

Visual inspection of the predicted trimer structures of lysozyme suggested a greater role for the residues of interest in interprotein interactions of amyloidogenic variants than WT lysozyme (Figure 2.4). When examined quantitatively, both amyloidogenic variants

had stronger and more numerous bonds between proteins compared to WT (Table 2.3). This suggests the residues of interest may be largely facilitating the predicted sharp increase in interprotein interactions, supporting the important role predicted for these residues by 3D structural analysis and network analysis.

A Role for Mutant Native Structure in Enhancing Lysozyme Amyloid Fibril Formation?

Taken together, these data support the hypothesis that residues 21, 62, 104, 112-117, and 122 in the mutant native states are involved in lysozyme amyloid primary nucleation. While the instability of amyloidogenic variants of lysozyme is almost certainly the most important factor for fibril formation (Dumoulin et al. 2005), it may not be the only factor involved. The greater interprotein interactions predicted to occur between trimeric amyloidogenic mutants compared to WT lysozyme may lead to amyloidogenic variants oligomerizing more readily before unfolding. We hypothesize these aggregates are composed of mostly mutant proteins because heterodimers with WT lysozyme have less interprotein bonding energies than mutant homodimers. This suggests when one of the lysozyme variants partially unfolds, it may already be in proximity to or bound by another mutant molecule, leading to a greater probability of amyloid nucleation. The sequence of events of the unfolding process for WT lysozyme and the I56T variant are consistent with our hypothesis. It has been demonstrated that lysozyme alpha helices A, B, and D (Moraitakis and Goodfellow 2003) are some of the last regions to unfold (Dhulesia et al. 2010). All the residues identified in this report except residue 62 are in or near regions of the protein that unfold later in the unfolding process, so these positions have a greater chance of maintaining their structure to facilitate intermolecular interactions during this

process. Residues 21, 62, 104, and 122 do not appear to be involved in increasing the flexibility of lysozyme in the amyloidogenic mutants because the total predicted bond energies from RING analysis for monomers do not suggest consistent and structurally important differences when compared to WT. Furthermore, the average *B*-factors for these residues in mutant lysozyme do not consistently differ from WT. It has been suggested that residues not present in the partially unfolded region can be altered without affecting the process of amyloid fibril formation (Ahn et al. 2012), but our data challenge this suggestion. It has been noted that lysozyme amyloid plaques are nearly entirely composed of the amyloidogenic variant free from WT protein (Morozova-Roche et al. 2000). This may be due not only to the greater instability of amyloidogenic proteins, but also due to the predicted favored intermolecular interactions of mutant homodimers and homotrimers compared to heterodimers with WT molecules (Table 2.4). A better understanding of the process of amyloidogenesis for lysozyme could yield insights into treatments for many different types of amyloidoses.

Study Limitations and Future Studies

Data from various computational approaches in this study support a role for lysozyme residues 21, 62, 104, 112-117, and 122 in lysozyme amyloidosis. However, this study has several limitations. The most obvious limitation is the small sample size of 3D structures used. Unfortunately, the study is limited by the availability of PDB files of human amyloidogenic lysozyme variants. We draw our conclusions from three PDB files, making this a preliminary study. The findings reported here may not be shared by all amyloidogenic variants of lysozyme. To increase confidence in our conclusions, further

studies should explore the structures of WT and amyloidogenic lysozyme under various experimental conditions. Furthermore, these hypotheses need to be experimentally tested to verify the importance of these residues for amyloidogenesis. Lysozyme mutants with smaller side chains or nonpolar side chains at the residues of interest could be created and the fibril formation kinetics studied. Data from studies of double mutants may also be useful. Future studies could be performed where residues 21, 62, 104, 112-117, or 122 are mutated in combination with I56T or D67H and tested for altered fibril formation kinetics. However, it may be most useful to mutate residue 21 and a residue in 112-117 or 122 because residues 32 through 108 have been shown to form the core of the lysozyme fibril (Frare et al. 2006). Therefore, mutating these residues may interfere with amyloid formation after unfolding.

Conclusions and Testable Hypotheses Generated

This preliminary study used a combination of 3D structural and residue interaction network analyses to support roles for residues 21, 62, 104, 112-117, and 122 in lysozyme amyloidosis. By comparing two amyloidogenic variants to WT lysozyme, we were able to identify network and 3D structural changes that were shared between the amyloidogenic variants. Modeling dimer and trimer interactions further supported a role for these residues. These residues appear to be especially important for trimer formation. This study generates several hypotheses that can be experimentally tested. 1) Residues 112-117 are less flexible in amyloidogenic variants of lysozyme than in the WT. 2) Residues 21, 62, 104, 112-117, and 122 are involved in the primary nucleation of lysozyme by facilitating intermolecular interactions between mutant lysozyme molecules. 3) Trimers of lysozyme

are more stable than dimers. 4) Lysozyme mutant molecules favor self-interactions over interactions with WT molecules. 5) Interprotein interactions in or close to the native state likely play a larger role in amyloid formation in general than previously hypothesized. This study demonstrates the utility of combining 3D structural and network analysis for understanding amyloid formation. Furthermore, it provides insight into lysozyme amyloid formation that may be applicable to the study of many other amyloidoses.

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CHAPTER 3

RESIDUE INTERACTION NETWORKS OF AMYLOIDOGENIC PROTEINS: POTENTIAL INSIGHTS INTO THE PRIMARY NUCLEATION OF AMYLOID-BETA

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Abstract

Amyloid plaques are known to contribute to the pathogenesis of a variety of diseases, but few 3D structural or sequence similarities among these proteins have been found. Here we report similarities in the residue interaction networks (RINs) of unrelated non-amyloid forms of amyloidogenic proteins. RINs for amyloidogenic proteins, non-amyloidogenic proteins, and random network controls were created, and various network metrics were calculated to test for differences in network structures. After finding differences in the RINs between a set of control proteins and a set of amyloidogenic proteins, we found differences in network structures when comparing amyloidogenic variants to non-amyloidogenic variants of the same proteins. Next, we focused on amyloid-beta (1-42) (Abeta42) because of its likely involvement in the pathogenesis of Alzheimer's disease (AD). Abeta42 is more amyloidogenic and therefore more likely to form amyloid plaques in aqueous environments than nonpolar environments that resemble cell membranes. We examined the network and structural differences in Abeta42 in a more polar versus a relatively nonpolar solvent and found some of the network features of amyloids were only present when Abeta was in the more polar environment. Insights from the combined use of network analysis and structural data suggest that the interaction between Val24 and Ile31 in Abeta42 in more nonpolar environments may be involved in preventing amyloidogenesis. This *in silico* study generates hypotheses that could provide insight into amyloid primary nucleation.

Keywords

Protein Stability, Alzheimer's Disease, Network Analysis, Network Centralization

Background

In many neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease, specific proteins are known to aggregate into cross-β sheet structures called amyloid fibrils (Makin and Serpell 2005). There are dozens of these diseases, collectively called amyloidoses, that affect several physiological systems (Greenwald and Riek 2010). Amyloid fibrils are thought to contribute to disease processes (Knowles et al. 2014). The protein monomers that form fibrils associated with diseases are diverse, and there are few structural and sequence similarities among them (Tzotzos and Doig 2010). Further understanding the similarities among amyloidogenic proteins that are present could provide insight into the first steps of nucleation, when two or three molecules of the same protein interact to form oligomers that may continue aggregating to form fibrils (Knowles et al. 2014). To investigate the molecular properties of amyloidogenic proteins that lead to plaque formation, we examined the residue interaction networks (RINs). RINs represent the residue interactions that are likely to occur as determined by the protein structure and are reviewed by Di Paola et al. (Di Paola et al. 2013). Each amino acid is represented as a node, and edges are drawn between residues that are likely to interact (**Figure 3.1**). Various metrics have been devised to mathematically describe the network size and the connections within a network (Estrada 2012), and many of these metrics have biological interpretations useful for analyzing protein structure (Amitai et al. 2004; Scardoni and Laudanna 2012) (**Table 3.1**). It has even been found that 3D structural

analysis techniques such as normal mode analysis reveal features that are largely determined by RINs (Bahar et al. 2010). RINs are powerful tools because they examine proteins from the perspective of a whole system rather than using a reductionist approach. This information complements more traditional structural and sequence studies, adding another layer to the understanding of protein function. RIN observations indicate residues that are important to the network (Estrada 2012); if a residue is important to the network, it is likely important to the 3D structure of the protein. This study investigates associations between RINs and amyloidogenicity to provide novel insights into common 3D structural features of non-amyloid forms of amyloidogenic proteins. If the non-amyloid forms of amyloidogenic proteins have similar features distinct from nonamyloidogenic proteins, these features may predispose the proteins to nucleate and fibrillize into amyloids.

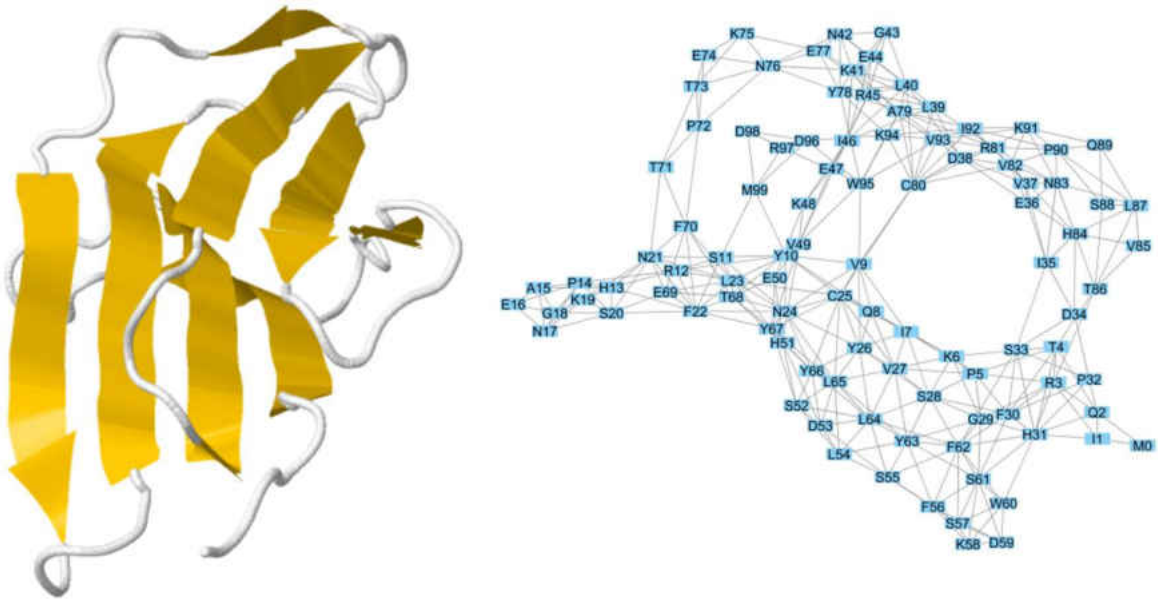

Figure 3.1: Three-dimensional and network structures of β-2-microglobulin. Residue interaction networks (RINs) abstract a 3D protein structure (left) into a network (right) of nodes (representing residues) and edges (representing potential residue bonds). This structure and network is for β-2-microglobulin (PDB ID: 5CSB). The numbering of residues in the network begins at zero in this figure only.

Table 3.1: Network metrics and their interpretations

Metric	Interpretation of Metric
Average Clustering Coefficient	Average connectedness of a node's neighbors
Centralization	Describes how much the network relies on hubs
Average Shortest Path Length	Mean minimum number of edges between one node and any other node
Density	Describes how connected the network is compared to a fully connected network
Average Number of Neighbors	Mean number of edges connected to a node
Highest MCODE Score	Describes the "strength" of the top cluster
Average of Top 3 MCODE Scores	Describes the average "strength" of the top three clusters
Clusters per Node	How many clusters are in a network (corrected for network size)
Number of Nodes	Total number of nodes in a network
Betweenness	Describes the extent to which a node is in the shortest path between other node pairs
Residue Centrality	Change in mean shortest path length after removing a node and its edges

Amyloid-beta (1-42) (Abeta42) is a 42 amino acid peptide that is hypothesized to be a causative factor in Alzheimer's disease (Hardy and Higgins 1992). As discussed above, Abeta42 forms amyloid fibrils and plaques in the brains of AD patients. Many drug candidates have been designed to reduce the amount of amyloid plaques in the AD brain, but these efforts have been largely ineffective in treating AD in human trials (Herrup 2015). If blocking oligomerization and amyloidogenesis of Abeta42 can lead to treatments

for AD, a better understanding of the primary nucleation of this peptide could lead to more effective drugs.

Abeta is a well-studied peptide, and there are many reports describing the relationship between fibrillization and the Abeta sequence. However, Abeta contains amino and carboxyl terminal regions of intrinsic disorder, and therefore detailed structural studies of the full-length peptide have proven challenging. The central hydrophobic cluster (CHC), consisting of residues 17-21, has been shown to be important for fibrillization (Wurth et al. 2002). Studies with fragments containing the CHC reveal that replacing any of the five residues prevents fibrillization (Wood et al. 1995). Several other studies have also found the CHC to be important for fibrillization (Hilbich et al. 1991; Hilbich et al. 1992; Esler et al. 1996; Wurth et al. 2002). However, the CHC is not the only region suspected to be involved in fibril formation. Scanning proline mutagenesis of Abeta40 identified residues 24-26 and 31-34 as important for fibrillization (Williams et al. 2004). The N-terminal 10 to 15 residues have not been found to have much effect on amyloid formation (Sánchez De Groot et al. 2005).

While some amyloidogenic proteins have regions that lack clearly defined structure, there is much evidence using different experimental conditions that Abeta is not completely disordered, and that the structured regions that are present play a role in nucleation and fibril formation. Studies have detected secondary structure in Abeta40 or Abeta42 dissolved in hexafluoroisopropanol (HFIP) (Crescenzi et al. 2002; Tomaselli et al. 2006), aqueous solvent with and without trifluoroethanol (TFE) (Soto et al. 1995), aqueous sodium dodecyl sulfate (SDS) micelles (Coles et al. 1998; Shao et al. 1999), aqueous urea solutions (Chen and Glabe 2006), and aqueous glycine solutions (Kirkitadze

et al. 2001). HFIP and aqueous SDS micelles are used to model a phospholipid membrane environment (Shao et al. 1999; Crescenzi et al. 2002), so protein structures in these solutions are likely similar to the *in vivo* membrane structures. Studies have shown that some secondary structure is necessary for Abeta amyloid formation because Abeta fibrillization was decreased following the near complete denaturation of Abeta by urea (Chen and Glabe 2006).

While the presence of secondary structure in Abeta dissolved in several different solvents has been described, there is not much consensus on the structural details. For example, while several studies have provided support for the existence of a turn region in Abeta, there are conflicting reports about where the turn occurs and even how many turns there are. These inconsistencies are likely due to different solvent conditions and peptide fragments used for these experiments. Williams et al. report two turn regions around residues 22-23 and 29-30 (Williams et al. 2004), whereas Morimoto et al. report only one turn around residues 22-23 (Morimoto et al. 2004), and Lazo et al. found evidence of one turn near residues 24-28 (Lazo et al. 2009). More data will likely help clarify these inconsistencies reported for Abeta structure, allowing for more precise drug targeting.

In this study we used both RINs and structural data to provide novel insights into the primary nucleation of Abeta42 and the role of solvent polarity in nucleation. We found that amyloidogenic proteins tend to be smaller, denser, and more centralized when compared to non-amyloidogenic controls. These features indicate networks that are more reliant on hubs. Potential binding pockets including these hub amino acids could prove useful as drug targets. Furthermore, our data suggest that when Abeta42 is present in a

more nonpolar solvent or membrane environment the interactions between Val24 and Ile31 in Abeta42 may play a role in preventing primary nucleation.

Methods

Creating Residue Interaction Networks from PDB Files

3D structure files for amyloidogenic proteins available in the Protein Data Bank (PDB) (Berman et al. 2000) (**Table 3.2**) and proteins not known to form amyloid fibrils in disease (Real Protein Controls, **Table 3.3**) were downloaded. The PDB files were uploaded to the Protein Graph Repository Converter (PGR Converter) (Dhifli and Diallo 2016 Jan 24) and used to create RINs. A node was considered to be the alpha carbon and an edge was drawn between every alpha carbon within 7 Å. The networks were exported from PGR Converter as GML files and then imported into Cytoscape v3.4.0 (Shannon et al. 2003) for visualization and network analysis.

Table 3.2: Network metrics of amyloidogenic proteins

PDB ID	Name	SCOP Class	Closest Match	Nodes	Density	Clusters per Node	Centralization
1ZEH	Insulin	Small Protein	α	102	0.074	0.069	0.056
1IYT	Amyloid-β (1-42)	Peptide	α	42	0.168	0.119	0.054
1QLX	Human Prion Protein	α+β	α+β	104	0.070	0.115	0.047
1XQ8	α-Synuclein	Coiled Coil	α	140	0.046	0.100	0.019
2KB8	Amylin	NA	α	37	0.188	0.108	0.095
1LOZ	Lysozyme (I56T)	α+β	α+β	130	0.060	0.092	0.049
4IP8	Serum Amyloid-α	α	α	420	0.021	0.074	0.015
5CSB	β-2-Microglobulin (D76N)	NA	β	100	0.073	0.090	0.049

Table 3.3: Network metrics of real protein controls

SCOP Protein Class	Control Number	PDB ID[a]	Nodes	Density	Clusters per Node	Centralization
α	1	1A6M	151	0.052	0.073	0.028
	2	1ALL	321	0.026	0.059	0.015
	3	1B33	2060	0.004	0.064	0.002
	4	1C75	71	0.114	0.099	0.103
	5	1DLW	116	0.067	0.086	0.038
	6	1DO1	153	0.052	0.092	0.034
	7	1DWT	152	0.052	0.079	0.035
	8	1FPO	499	0.015	0.060	0.009
	9	1FXK	349	0.023	0.049	0.012
	10	1G08	572	0.014	0.068	0.009
	11	1H97	294	0.028	0.071	0.017
	12	1HBR	572	0.014	0.072	0.009
	13	1IDR	253	0.030	0.087	0.022
	14	1IRD	287	0.028	0.077	0.017
	15	1KR7	110	0.070	0.091	0.041
	16	1KPT	334	0.025	0.078	0.014
	17	1LIA	664	0.012	0.062	0.009
	18	1MWC	306	0.026	0.059	0.017
	19	1NEK	1068	0.008	0.073	0.005
	20	1PHN	334	0.024	0.072	0.015
α+β	21	1ALC, 1AVP	168.5	0.051	0.105	0.036
	22	1BRN, 1CNS	351	0.026	0.081	0.016
	23	1CQD, 1EUV	582	0.018	0.079	0.013
	24	1F13, 1GCB	946.5	0.012	0.082	0.008
	25	1GOU, 1IWD	216.5	0.036	0.081	0.027
	26	1K3B, 1LNI	272	0.031	0.098	0.025
	27	1LSD, 1ME4, 1MZ8, 1PPN	245	0.039	0.101	0.031
	28	1QMY, 1QSA, 1UCH, 2ACT	377.5	0.026	0.084	0.019
β	29	1AUN, 1BEH, 1BHU, 1DMH, 1DO6	308.6	0.039	0.090	0.030
	30	1F35, 1F86, 1G13, 1HOE, 1I9R	569	0.038	0.091	0.023
	31	1IAZ, 1IFR, 1JK6, 1KCL, 1KNB	299	0.041	0.097	0.030
	32	1SFP, 1SHS, 1SLU, 2MCM, 2HFT	338.4	0.042	0.082	0.033

Creating Real Protein Controls

The proteins selected for the real protein controls were based on the protein control groups created by Bagler (Bagler and Sinha 2005; Bagler 2006) and Sinha (Bagler and Sinha 2005). The Bagler and Sinha protein library consists of 20 proteins each of the α, β, α+β, and α/β structural classes as defined by the Structural Classification of Proteins (SCOP) (Murzin et al. 1995) for a total of 80 protein control entries. We searched the amyloidogenic proteins in SCOP, and, if they were assigned to a protein structure class that was not α, β, α+β, or α/β, we classified them as α, β, α+β, or α/β based on the closest best fit determined by visual inspection (Table 3.2). If a protein was not included in SCOP, we searched for structural matches in SCOP and used the class of the closest match. The resulting amyloidogenic group was 62.5 % α-class, 12.5 % β-class, 25 % α+β-class, and 0 % α/β class. The real protein control group was adjusted to reflect the structural class composition of the amyloidogenic group by averaging metrics from random subgroups of real protein control entries in the same class to condense the 20 protein entries in each group so we could create a control group with 20 entries from the α-class, 4 entries from the β-class, and 8 entries from the α+β-class. This method yielded a control group with the same percent composition of protein classes as the amyloidogenic protein group. The averaged random subgroups and the resulting control group are included in Table 3.3.

Creating Random RIN Controls

The RIN metrics from the amyloidogenic proteins were compared to the real protein controls. However, the real protein controls generally consisted of larger networks (Table 3.3). To determine if differences between groups are due to network size or network

connections, we created a random network control group of the amyloidogenic proteins (**Table 3.4**). To create the random protein controls, we retained the number of nodes and edges from each RIN for the amyloidogenic proteins, but the connections between them were randomized using the Network Randomizer application (Tosadori et al. 2016) in Cytoscape. For each amyloidogenic protein RIN, fifty random networks were created. The RIN metrics were calculated for each of the networks, and then the mean of the fifty resulting metric values for each type of metric was used as the randomized metric value for the random control of a single amyloidogenic protein. This process was repeated for each amyloidogenic protein.

Table 3.4: Average metrics of randomized networks of amyloidogenic proteins

PDB ID	Name	Nodes	Density	Clusters per Node	Centralization
1ZEH	Insulin	102	0.074	0.033	0.074
1IYT	Amyloid-β (1-42)	42	0.168	0.056	0.142
1QLX	Human Prion Protein	104	0.070	0.031	0.070
1XQ8	α-Synuclein	140	0.046	0.019	0.051
2KB8	Amylin	37	0.188	0.056	0.154
1LOZ	Lysozyme (I56T)	130	0.060	0.028	0.062
4IP8	Serum Amyloid-α	420	0.021	0.011	0.024
5CSB	β-2-Microglobulin (D76N)	100	0.073	0.033	0.071

Calculating RIN Metrics

Local RIN metrics were calculated in Cytoscape using the Network Analyzer application (Assenov et al. 2008). The MCODE application (Bader and Hogue 2003) in Cytoscape was used to calculate the number of clusters in a network. Centrality measures of Abeta42 included betweenness and residue centrality. Residue centrality was calculated

as by Hu et al. (Hu et al. 2014) by taking the average path length for the whole Abeta42 network, deleting a single node, recalculating the average path length for the resulting network, and then finding the absolute value of the change in average path length from the whole network. This was repeated for every node in the Abeta42 network. These values were used to determine which nodes have the highest betweenness values and which nodes are the most critical for maintaining the average path length. We then calculated betweenness values for residues in Abeta42 and Abeta40 in more polar and more nonpolar solvents (PDB structure files 1IYT (Crescenzi et al. 2002); 1Z0Q (Tomaselli et al. 2006); 1BA4 (Coles et al. 1998); 2LFM (Vivekanandan et al. 2011)). Next, we averaged the betweenness values for residue regions of interest in Abeta and compared them across all four structures.

Overlapping Abeta42 Structured Regions and Analyzing Residue Interactions

The PDB files for Abeta42 were visualized using UCSF Chimera v1.11.2 (Pettersen et al. 2004). The unstructured regions of each of the PDB files as determined by visual inspection (residues 1-9 and 33-42) were trimmed off, and then the two structures were overlapped using the MatchMaker application (Meng et al. 2006) in UCSF Chimera. The structural data for the PDB files for Abeta42 are from NMR experiments, so there is information for multiple models in each file. To determine the residue interactions occurring in each model, each chain was individually input into the Residue Interaction Network Generator (RING) (Piovesan et al. 2016), and networks were created using the alpha carbons as nodes and by drawing a single undirected edge for each type of interaction between two residues. The resulting networks were each analyzed for the

presence of edges between the residues of interest identified from the betweenness and residue centrality measures.

Analyzing Predicted Abeta42 Trimer Interactions

To determine the potential differences of contributions of the residues of interest to the energy of interpeptide bonding in a solvent, we used ClusPro (Kozakov et al. 2017) to predict the trimer structure of Abeta42 in a nonpolar or a polar solvent. The resulting PDB file for the ClusPro top-rated predicted structure was input into RING, and networks were created as described above. Bonding information was exported to Microsoft Excel and the predicted bond energies for interpeptide interactions were summed for each condition.

Statistical Analysis of Data

All statistical analyses were completed with GraphPad Prism v7.0, and p-values $<$ 0.05 were considered significant.

Comparing Amyloidogenic Proteins, Real Protein Controls, and Random Network Controls. The data was tested for normality using the D'Agostino-Pearson normality test. When the data distribution was normal, and the groups had equal variance as determined by Bartlett's test, a one-way ANOVA with Tukey's *post hoc* test was performed. This was the case for comparing the RIN metric clusters per node. If data was not normally distributed but had equal variance, the Kruskal-Wallis test with Dunn's multiple comparisons was performed. This was the case for number of nodes and centralization. If data was not normal and did not have equal variance, it was transformed by taking the

square root of each value and then rechecked for normality. We then performed a one-way ANOVA with Tukey's *post hoc* test. This was the case for network density.

Analyzing Abeta42 Centrality Metrics. To determine which residues had high centrality values, we checked the data for normality using the D'Agostino-Pearson normality test before calculating the z-score for each of the residues. Betweenness values were normalized by taking the square root of each value, and residue centrality values were normalized by taking the cube root. Z-score absolute values ≥ 2 were considered statistically significant. For both betweenness and residue centrality, Val24 and Ile31 had z-score absolute values ≥ 2.

Comparing Average Betweenness Values of Regions in Abeta. The arithmetic mean for betweenness was calculated for regions of interest of different lengths across several PDB structures of Abeta. These average betweenness values of regions were compared to the average betweenness of the entire structure using a paired one-way ANOVA with Tukey's *post hoc* test.

Results

RINs of Amyloidogenic Proteins Have Unique Features Compared to Controls

Of the RIN metrics analyzed (average clustering coefficient, centralization, average shortest path length, density, average number of neighbors, highest MCODE cluster score, average of top 3 MCODE cluster scores, clusters per node, and number of nodes), four metrics showed significant differences when comparing amyloidogenic proteins to the

controls (**Figure 3.2**). The eight amyloidogenic proteins in our data set are shown in Table 3.2. If the amyloidogenic protein group differs from the real protein controls in the same direction and magnitude as the random controls, then the differences are due to network size and not wiring (the specific connections between nodes). If both the amyloidogenic protein group and the random control group differ from the real protein controls and differ from each other, then at least some of the differences are due to network wiring. First, compared to real protein controls, amyloidogenic proteins tend to have fewer nodes (Figure 3.2A) and a higher density (Figure 3.2B). These metrics are a measure of network size rather than connections. However, some measures related to network wiring also showed significant differences. Amyloidogenic proteins had 1) slightly greater numbers of clusters per node (Figure 3.2C) compared to real protein controls or random networks. Furthermore, 2) the centralization of amyloidogenic RINs was greater than real protein controls but less than random network controls (Figure 3.2D).

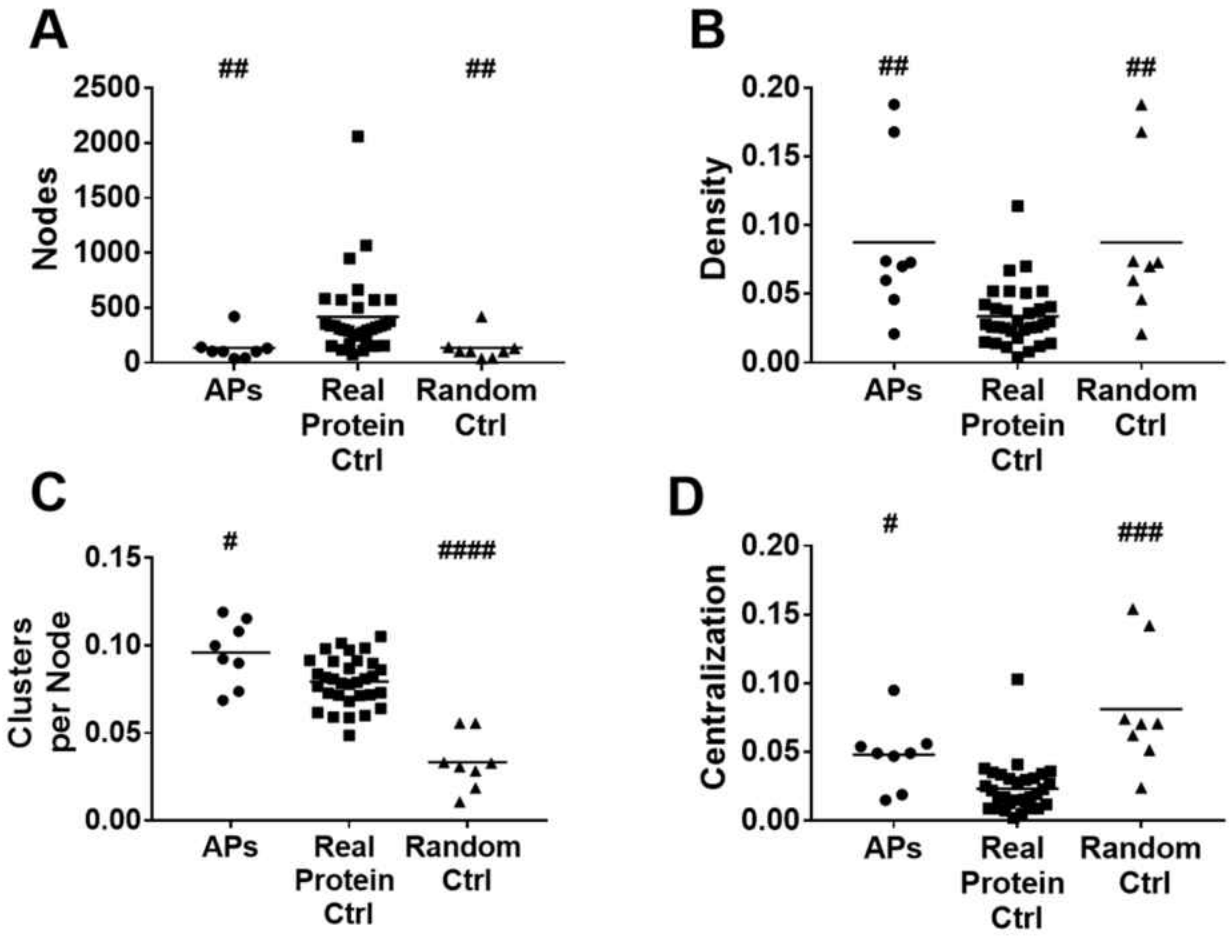

Figure 3.2: Comparing network metrics for amyloidogenic proteins (APs), real protein controls (Real Protein Ctrl), and random network controls (Random Ctrl). **#** indicates $p \leq 0.05$, **##** indicates $p \leq 0.01$, **###** indicates $p \leq 0.001$, and **####** indicates $p \leq 0.0001$ when compared to Real Protein Ctrl.

RINs of Amyloidogenic Variants of Proteins Show Similar Patterns as When Comparing Amyloidogenic RINs to Real Protein Controls

We compared the RINs of wild type (WT) variants to amyloidogenic variants of the same proteins. The findings are reported in **Table 3.5** for lysozyme (an α+β class protein) and **Table 3.6** for β-2-microglobulin (a β-class protein). Lysozyme and β-2-microglobulin

were chosen because they both had WT and amyloidogenic variant structures available in the Protein Data Bank (Muraki et al. 1996; Booth et al. 1997). For centralization but not for density or clusters per node the amyloidogenic lysozyme variants I56T and D67H showed similar differences from the WT protein as the group of amyloidogenic proteins showed when compared to real protein controls. For centralization and density only D76N β-2-microglobulin showed similar differences from the WT protein as the group of amyloidogenic proteins showed when compared to real protein controls.

Table 3.5: Effects of amyloidogenic mutations of lysozyme (α+β class) on RIN metrics

Network Metric	**WT[a]**	**I56T[b]**	**D67H[c]**	**Consistent with Group Trends?**
Centralization	0.049	0.049	0.050	Yes
Density	0.0604	0.0602	0.0598	No
Clusters per Node	0.108	0.092	0.115	No

[a]PDB ID 1REX
[b]PDB ID 1LOZ
[c]PDB ID 1LYY

Table 3.6: Effects of amyloidogenic mutations of β-2-microglobulin (β-class) on RIN metrics

Network Metric	**WT[a]**	**D76N[b]**	**Consistent with Group Trends?**
Centralization	0.040	0.049	Yes
Density	0.072	0.073	Yes
Clusters per Node	0.10	0.09	No

[a]PDB ID 5CS7
[b]PDB ID 5CSB

RINs of Abeta40 and Abeta42 Show Similar Patterns as When Comparing Amyloidogenic RINs to Real Protein Controls

We compared the RINs from Abeta42 in 20% H_2O, 80% hexafluoroisopropanol (v/v) (Crescenzi et al. 2002), a more nonpolar solvent (predicted to make Abeta less amyloidogenic), to those in 70% H_2O, 30% hexafluoroisopropanol (v/v) (Tomaselli et al. 2006), a more polar solvent (predicted to make Abeta more amyloidogenic), and found that the centralization was different and changed in a direction consistent with the trends described above for real protein controls versus amyloidogenic proteins. However, clusters per node or density did not follow these trends (**Table 3.7**). We found similar results when comparing the RINs of Abeta40 in nonpolar and polar solvents (**Table 3.8**).

Table 3.7: Effects of solvent conditions on amyloid-beta (1-42) RIN metrics[a]

Network Metric	Nonpolar[b]	Polar[c]	Consistent with Group Trends?
Centralization	0.054	0.063	Yes
Density	0.168	0.159	No
Clusters per Node	0.119	0.095	No

[a] Amyloid-beta (1-42) tends to form amyloid plaques in polar solvents (Crescenzi et al. 2002).
[b] PDB ID 1IYT
[c] PDB ID 1Z0Q

Table 3.8: Effects of solvent conditions on amyloid-beta (1-40) RIN metrics[a]

Network Metric	Nonpolar[b]	Polar[c]	Consistent with Group Trends?
Centralization	0.069	0.086	Yes
Density	0.165	0.123	No
Clusters per Node	0.125	0.125	No

[a] Amyloid-beta (1-42) tends to form amyloid plaques in polar solvents (Crescenzi et al. 2002).
[b] PDB ID 1BA4
[c] PDB ID 2LFM

RINs of Abeta40 and Abeta42 Reveal Higher Mean Betweenness Values for the CHC

The central hydrophobic cluster (CHC) in the Abeta40 and Abeta42 peptides spans residues 17-21. Compared to the average betweenness of each of the entire structures, the CHC had a significantly higher average betweenness (**Figure 3.3**). Because betweenness can indicate structurally important residues, this agreed with the visual examination of the 3D structure of Abeta40 and Abeta42 in each solvent where it is visually evident that the CHC largely retains its structure.

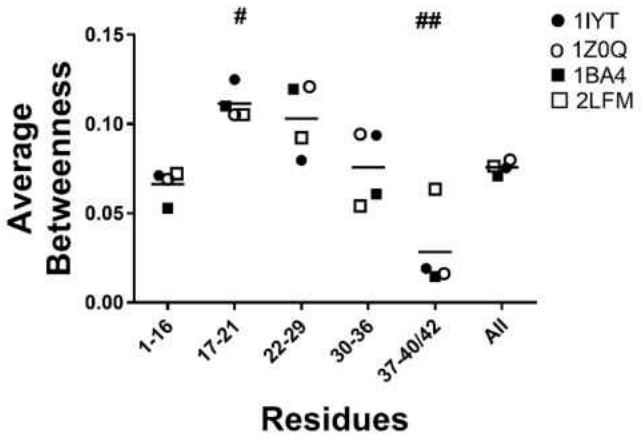

Figure 3.3: Average betweenness of regions of Abeta40 and Abeta42 compared to the average betweenness of the entire node set. # indicates $p \leq 0.05$ and ## indicates $p \leq 0.01$.

Val24 and Ile31 Have Significantly Higher Centrality Values

The betweenness (**Figure 3.4A**) and residue centrality (**Figure 3.4B**) measures for each residue in Abeta42 in the more nonpolar solvent are shown. In each case, the values for Val24 and Ile31 were higher than the value for the average node in the network, and

each has a z-score absolute value of ≥ 2.0, so they were significantly different from the values of the other residues. Structural data indicated that Val24 and Ile31 were likely to form hydrophobic interactions in 3 of 10 NMR models in the PDB 1IYT structure file (more nonpolar solvent), but no hydrophobic interactions between these residues were predicted in any of the 30 models in the PBD 1Z0Q structure file (more polar solvent). The structures in the more nonpolar and more polar environments for representative NMR models are shown in **Figure 3.5**.

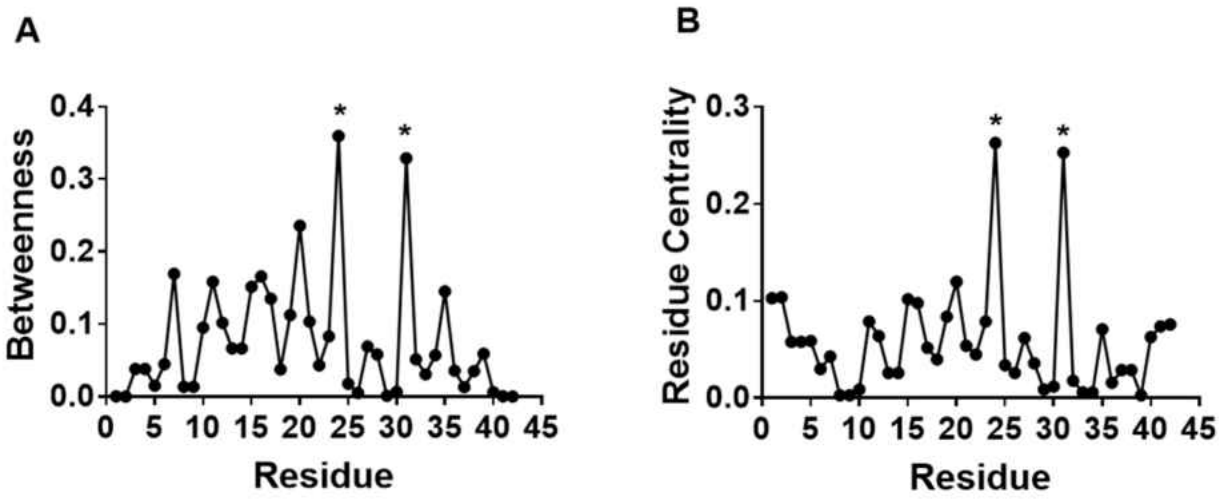

Figure 3.4: Betweenness and residue centrality values for each residue in Abeta42 in a more nonpolar solvent (1IYT). * indicates a z-score ≥ 2.0.

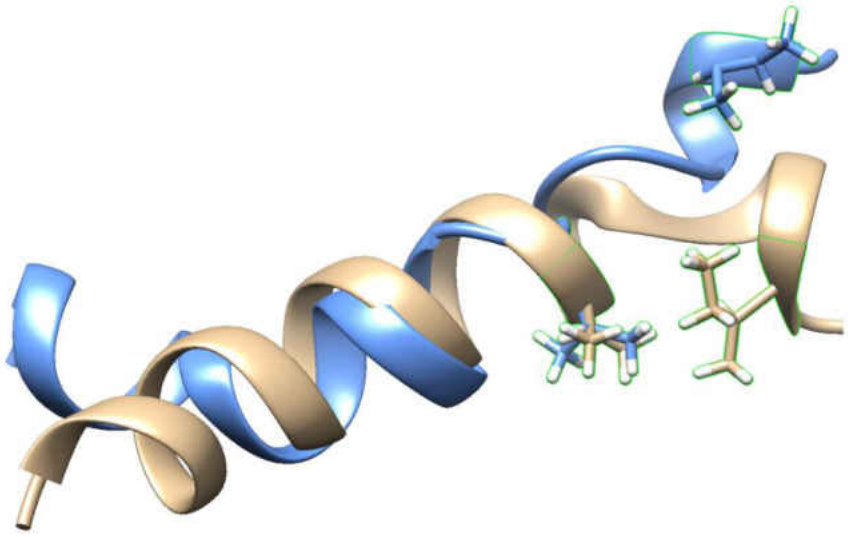

Figure 3.5: Structured regions of Abeta42 in a more nonpolar solvent (tan, 1IYT) and a more polar solvent (blue, 1Z0Q). The side chains of Val24 and Ile31 are shown highlighted in green in each case.

Predicted Trimers of Abeta42 Suggest Greater Interpeptide Bonding Energy in More Polar Solvents

The PDB files of the predicted trimers with the top ClusPro ranking for 1IYT (showing the possible Val24-Ile31 hydrophobic interaction) and 1Z0Q (showing no Val24-Ile31 hydrophobic interaction) were used to generate RINs, and the predicted bonding energies were analyzed (**Figure 3.6**). The total bonding energy between chains was larger for the predicted trimer with no hydrophobic Val24-Ile31 interaction (1Z0Q PDB file).

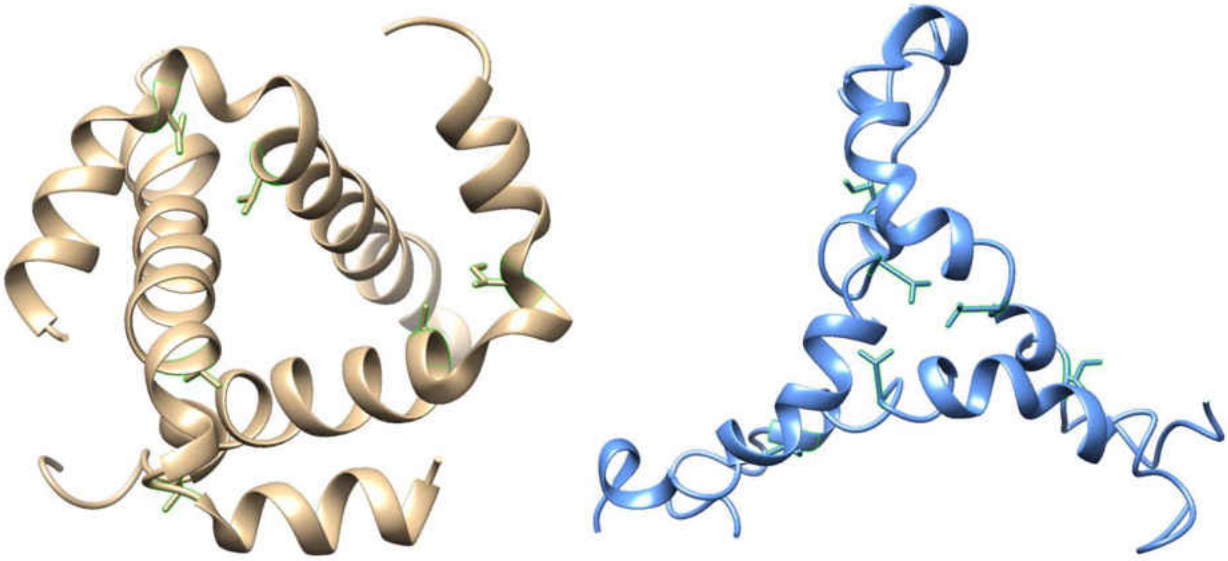

Figure 3.6: Predicted trimer structures of Abeta42. UCSF Chimera predictions of trimer formation for Abeta42 in a more nonpolar solvent (left, 1IYT) and a more polar solvent (right, 1Z0Q). The side chains of Val24 and Ile31 are shown highlighted in green in each case.

Discussion

RINs From Amyloidogenic Proteins Suggest Similarities in Structure

By comparing non-amyloidogenic protein RINs to those of amyloidogenic proteins, differences unique to amyloidogenic proteins were detected. When interpreted in the light of structural data, the results could be important for understanding the amyloidogenic process and developing strategies to prevent it. RIN metrics have important biological interpretations, allowing for structural and functional predictions (Amitai et al. 2004; Scardoni and Laudanna 2012). Compared to real protein controls, amyloidogenic proteins tended to be more centralized, smaller, denser, and had more clusters per node (Figure 3.2). The network metric differences due to differences in network wiring provide novel

insight into the structure of amyloids. Amyloidogenic proteins had more clusters per node. A cluster is a group of nodes that is highly connected (Bader and Hogue 2003), indicating the potential for many residue interactions. This suggests a greater number of local areas of higher stability in amyloidogenic proteins compared to non-amyloidogenic proteins. This does not contradict the well-established finding that destabilizing mutations increase amyloidogenesis because the amyloidogenic proteins could be globally less stable while having a few local areas of higher stability. Amyloidogenic proteins were also more centralized. Centralization describes the "star-like" character of a network (Pavlopoulos et al. 2011), which can also be described as reliance on hubs. A reliance on hubs makes proteins more robust against random "attacks" such as random amino acid substitutions caused by DNA mutations, but it leaves them more susceptible to targeted attacks (Albert et al. 2000; Oltvai et al. 2000), such as those that can occur when groups of amino acids including hubs are targeted by rational drug design. Drugs targeting these hub regions are more likely to greatly disrupt or stabilize peptide structure than drugs targeting other regions of the protein. Network metrics revealed common features in amyloidogenic proteins that have not previously been described. These common features may enhance our understanding of the pathogenesis of amyloidoses.

Some of the Network Differences Observed for Amyloidogenic Proteins as a Group Are Robust Enough to Be Observed in Mutant Variants of the Same Protein

This study reports significant network differences between an amyloidogenic protein group and a group of non-amyloidogenic proteins. We also investigated whether the differences between amyloidogenic proteins and real protein controls held when

examining different mutant variants of the same protein. Lysozyme (an α+β class protein) has been extensively studied as a model of amyloidogenesis because it has mutant variants that differ by a single residue that are much more likely to form amyloid plaques (Swaminathan et al. 2011). We compared WT lysozyme network metrics to those of two different amyloidogenic variants and observed similar changes in centralization as when comparing amyloidogenic proteins to real protein controls (Table 3.5). The protein β-2-microglobulin (a β-class protein) also had a WT structure and an amyloidogenic variant for comparison. The expected trends held for centralization and density for β-2-microglobulin (Table 3.6). The network metric differences were understandably small because we analyzed networks that only had a single amino acid change. Because increased centralization was present in the amyloidogenic variants of proteins from these two classes, increased centralization may be a more robust difference between amyloidogenic proteins and non-amyloidogenic proteins than other network metrics tested. Therefore, drugs could be designed to target the regions of the peptide that include the hubs that amyloidogenic proteins appear to rely on most heavily for network structure.

Network Variations Can Be Observed for the Same Protein in Different Solvents

Some of the differences observed when comparing amyloidogenic proteins to real protein controls were observed even when comparing the networks of the same protein in different solvents. Abeta42 structures in HFIP were used because HFIP is a model for hydrophobic membrane environments (Crescenzi et al. 2002). While HFIP likely exaggerates the alpha helicity of Abeta (Pachahara et al. 2015), it is still a relevant model for Abeta structure *in vivo* because alpha helical Abeta likely plays a role in

amyloidogenesis (see Background). The two concentrations of HFIP used for structural studies also allow for a direct comparison to determine the effects of solvent polarity on Abeta structure. We examined network properties of Abeta42 dissolved in a more nonpolar solvent where it is less amyloidogenic and those present when it is dissolved in a more polar solvent where it is more amyloidogenic (Crescenzi et al. 2002). Interestingly, we found the Abeta42 network when the more polar solvent was used to be more centralized than that for Abeta42 when the more nonpolar solvent was used (Table 3.7). We observed the same trend when comparing Abeta40 dissolved in the more nonpolar and more polar solvents (Table 3.8). Because this trend was also found when comparing the amyloidogenic variants of lysozyme and β-2-microglobulin with their WT variants, increased centralization appears to be a consistent feature of amyloidogenic proteins. As discussed above, increased centralization could indicate the presence of effective drug targets in amyloidogenic proteins because of the increased reliance on hubs.

Results from RIN Metrics Support a Role for the CHC in Abeta Fibrillization

Amyloid-beta is a well-studied protein with much known about its structure. The CHC has been identified as an important region of Abeta for fibrillogenesis (Wurth et al. 2002). Consistent with these studies, we found that the CHC has significantly higher average betweenness values compared to the average betweenness values of the entire networks (Figure 3.3). Betweenness describes the frequency with which a node occurs in the shortest path length between any two other nodes (Chakrabarty and Parekh 2016). Betweenness is used as a metric to identify residues that are important for the overall network (Steuer and Lopez 2008) and therefore for the 3D structure. The significantly

higher average betweenness values for the CHC indicate that it is an important region for the structure of Abeta monomers. This agrees well with several other studies (Hilbich et al. 1991; Hilbich et al. 1992; Esler et al. 1996; Wurth et al. 2002), providing support for the usefulness of RINs in the study of protein structure and function.

Disrupting the Predicted Interaction Between Val24 and Ile31 in Abeta42 in a More Nonpolar Solvent May Facilitate Primary Nucleation

Because increased centralization suggests reliance on hubs, we analyzed network and structural data to identify the key residues for network structure of Abeta42 when dissolved in a more nonpolar solvent where it is less amyloidogenic. We calculated the betweenness and residue centrality values for each residue in the structure (Figure 3.4), and residues Val24 and Ile31 were shown to have significantly higher values than the other residues. Therefore, two separate methods for identifying key residues support the conclusion that the interaction between Val24 and Ile31 is important for overall network structure in Abeta42 when it is dissolved in a more nonpolar solvent.

Residue interaction network data is most useful when combined with sequence and structural data (Amitai et al. 2004), so we examined the 3D structure of Abeta42 when it is dissolved in a more nonpolar solvent to determine the effects that these residues may exert on the overall structure (Figure 3.5). When dissolved in the more nonpolar solvent, Abeta42had a noticeable turn near Val24 and Ile31, so we hypothesized the presence of a hydrophobic interaction between them. To test this hypothesis, we uploaded the PDB file to RING which predicted the interactions that occur within the structures. The predicted interaction between Val24 and Ile31 in a nonpolar environment may be important for

maintaining the less-amyloidogenic structure of Abeta42. The involvement of Val24 and Ile31 in Abeta fibrillization has previously been identified by scanning proline mutagenesis (Williams et al. 2004). To our knowledge, we are the first to predict an interaction between Val24 and Ile31 in Abeta42 to be important for preventing fibrillization.

To further determine why Abeta42 is more likely to engage in primary nucleation in more polar solvents compared to more nonpolar solvents, software was used to predict Abeta42 trimer formation under both solvent conditions, and interpeptide bonding energy was calculated (Figure 3.6). Evidence has shown that oligomers are the form of Abeta that is most toxic to neurons (Sakono and Zako 2010), so even if trimers only exist transiently on the path to fibril formation, structural studies of trimers are important. However, there is experimental evidence that relatively stable trimers and tetramers of Abeta exist (Chen and Glabe 2006). The interpeptide bonding energy was much higher for Abeta42 when dissolved in the more polar solvent. Increased bonding energy suggests tighter binding between the individual components of an Abeta42 trimer when dissolved in the polar solvent compared to the nonpolar solvent. We hypothesize that the lack of an interaction between Val24 and Ile31 allows for a more linear conformation, facilitating tighter bonding and primary nucleation. Therefore, drugs designed to stabilize the interaction between Val24 and Ile31 may prevent primary nucleation and reduce the number of toxic Abeta42 oligomers.

Study Limitations

While there are several dozen amyloidogenic proteins in the human proteome, our experimental group only contained eight due to the limited availability of PDB data files.

The relatively small size of the amyloidogenic protein group is a limitation of this study. Second, our conclusions regarding Abeta42 structural differences are based on only two structures. More structural data for Abeta42 in monomeric form in different solvent conditions would increase our confidence in the conclusions. Third, some of the effect sizes are relatively small. It is not entirely clear if these small effects could lead to functional differences between the groups. Finally, the hypotheses generated from the data presented here need to be experimentally tested to verify the validity of our conclusions. Sciarretta and colleagues created a lactam link between two different residues of Abeta40 to test the effects of conformational changes on the nucleation of beta-amyloid (Sciarretta et al. 2005). A similar method covalently linking Val24 to Ile31 in an aqueous solvent and comparing nucleation to an unlinked peptide could be used to test our hypothesis. However, the predicted Val24-Ile31 interaction is likely only one of several interactions necessary to prevent oligomerization.

Conclusions

Metrics from residue interaction network structures of amyloidogenic proteins were statistically different from those of a group of non-amyloidogenic control proteins. These differences included increased centralization and clusters per node. Increased centralization implies the presence of residues that act as hubs in the network, making the protein more susceptible to targeted attacks such as those occurring from rationally designed drugs. Some of these differences were observed even when comparing amyloidogenic variants to the WT non-amyloidogenic variant of the same protein. Furthermore, increased centralization was observed when comparing the structure of

Abeta42 in a more nonpolar solvent to that in the more amyloid-friendly polar solvent. The local RIN metrics of betweenness and residue centrality identified residues 24 and 31 as hubs. Combining this data with structural analysis suggests stabilizing the interaction between Val24 and Ile31 may help prevent oligomerization of Abeta42. Combining the use of network analysis and structural data produces novel insight that can be used for the design of drugs for the treatment of amyloidoses.

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CHAPTER 4

EFFECTS OF HIGH PROTEIN DIET AND LIVER DISEASE IN AN *IN SILICO* MODEL OF HUMAN AMMONIA METABOLISM: POTENTIAL IMPLICATIONS FOR HEPATIC ENCEPHALOPATHY

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Abstract

After proteolysis, the majority of released free amino acids from dietary protein are transported to the liver for gluconeogenesis or to peripheral tissues where they are used for protein synthesis and eventually catabolized, producing ammonia as a byproduct. High ammonia levels in the brain are a major contributor to the decreased neural function that occurs in several pathological conditions such as hepatic encephalopathy. Therefore, it is important to gain a deeper understanding of human ammonia metabolism. The objective of this study was to predict changes in blood ammonia levels resulting from alterations in dietary protein intake or from liver disease and to determine the toxicity of ammonia on differentiated SH-SY5Y neuroblastoma cells. A simple mathematical model was created using MATLAB SimBiology and data from published studies. Simulations were performed and results analyzed to determine steady state changes in ammonia levels resulting from varying dietary protein intake and varying liver enzyme activity levels to simulate liver disease. As a toxicity reference, viability was measured in SH-SY5Y neuroblastoma cells following differentiation and ammonium chloride treatment. Results from simulations yielded physiological steady state blood ammonia levels and reasonably approximated pathophysiological steady state levels resulting from liver cirrhosis or a genetic carbamoyl phosphate synthetase I (CPS1) deficiency. A pathophysiological level of ammonium chloride (90 μM) decreased the viability of differentiated SH-SY5Y cells by 14%. Data from the model suggest decreasing protein consumption may be one simple strategy to decrease blood ammonia levels and minimize the risk of developing encephalopathy for some liver disease patients.

Keywords

Ammonia, Hepatic Encephalopathy, Liver Cirrhosis, Carbamoyl Phosphate Synthetase 1, Nitrogen, Urea Cycle

Background

Protein is an abundant part of the human diet. It is recommended that humans consume 0.8 g of protein per kg body mass per day. For a male of average weight (88.7 kg) (Fryar et al. 2012), this is equivalent to 71 g of protein per day. When amino acids are consumed at a faster rate than they are used for protein synthesis, they are metabolized as an energy source, typically accounting for roughly 15%-20% of the energy supply. The liver breaks down nearly half of the amino acids in the human diet as substrates for gluconeogenesis (Jungas et al. 1992). Amino acid catabolism first relies upon the transfer of the amino group by aminotransferases to a ketoacid, often to alpha-ketoglutarate to form glutamate, and then by the deamination of glutamate by glutamate dehydrogenase which produces ammonia (NH_3). Roughly 12.5% of nitrogen intake is excreted from the digestive tract (Tome and Bos 2000). Because ammonia is relatively toxic (Auron and Brophy 2012), systems such as the urea cycle are in place primarily in the liver to convert it into a less toxic form that can be readily removed from the circulation and excreted.

The liver is the main organ responsible for filtering ammonia and other nitrogen sources such as glutamine from the blood to synthesize urea, the major form of excreted nitrogen in mammals. Urea is a relatively nontoxic waste product that safely stores nitrogen until it can be removed from the body. However, when ammonia is not successfully removed from the blood due to impaired or overwhelmed removal

mechanisms, the plasma ammonia concentration increases, which may cause deleterious effects such as neural impairment (Ott and Vilstrup 2014). As part of the process of nitrogenous waste removal, nitrogen-rich blood enters through the hepatic portal vein and is eventually filtered through the acinus, the functional unit of the liver, before draining out of the central vein. The acinus is divided into three zones (Brosnan and Brosnan 2009). Zone 1 is the closest to the hepatic portal vein, and zone 3 is the closest to the central vein. The hepatocytes spanning these three zones do not all perform the same metabolic functions (Häussinger et al. 1992); rather, different branches of nitrogen metabolism are localized to specific zones. Zones 1 and 2 contain the enzymes of the urea cycle (Moorman et al. 1989) as well as glutaminase (Moorman et al. 1994), an enzyme that removes nitrogen from glutamine to yield ammonia and glutamate. However, zone 2 has less glutaminase activity than zone 1. Zone 3 contains glutamine synthetase (Gebhardt and Mecke 1983), an enzyme that combines ammonia and glutamate to produce glutamine and is also called glutamate-ammonia ligase (GLUL).

Liver disease can change the activities of several key enzymes involved in nitrogen metabolism. For example, liver cirrhosis results in decreased expression of GLUL and the urea cycle enzyme CPS1 (Gebhardt and Reichen 1994; Fleming and Wanless 2013). Mutations in the *CPS1* gene can lead to individuals born with a deficiency in mitochondrial carbamoyl phosphate synthetase activity (Klaus et al. 2009). Because CPS1 catalyzes the first committed step of the urea cycle, this can have serious consequences on nitrogen metabolism. The increase in the number of individuals with liver disease in recent years (Mokdad et al. 2014) combined with an average protein intake in the U.S. that is about 40%

above the recommended value (NHAHES 2006) creates the need for understanding the effects of increased protein intake on blood ammonia levels.

Increased blood ammonia levels are a causative agent in hepatic encephalopathy (HE) (Butterworth 2003), but increased ammonia levels have also been implicated in other neural disorders such as Alzheimer's disease (Seiler 2002), amyotrophic lateral sclerosis (Parekh 2015), and Huntington's disease (Chen et al. 2015). HE results from liver damage leading to cognitive impairment. Liver disease also increases the blood levels of other potentially neurotoxic factors such as manganese and pro-inflammatory cytokines (Butterworth 2015) that may contribute to the encephalopathy as well. Between 30% and 45% (Poordad 2007) of the more than 600,000 patients (Scaglione et al. 2015) with liver cirrhosis each year will develop hepatic encephalopathy, resulting in a cost of nearly $1 billion per year (Poordad 2007). Most treatments aim to reduce the level of circulating ammonia (Butterworth 2003). Due to challenges in reliably assaying ammonia due to its reactivity (Goggs et al. 2008; Blanco Vela and Bosques Padilla 2011), there are not many studies that measured the effects of dietary alterations on blood or tissue ammonia levels. Our model provides further insight into how changes in dietary protein intake may affect blood ammonia levels to better direct these treatment strategies.

We used data from the literature to create a computational model that simulates ammonia metabolism and predicts blood ammonia levels based upon the amount of protein consumed and upon the degree of liver dysfunction. Results from the model agree relatively well with measured physiological and pathophysiological steady state metabolite levels, and several insights were made from varying our initial conditions to investigate the role of key enzymes in human organismal nitrogen metabolism. Cell culture studies were

used to extend the model and establish the toxicity of pathophysiological concentrations of ammonia on differentiated SH-SY5Y neuroblastoma cells in culture.

Methods

Description of the Model

This model describes the changes in ammonia, urea, and glutamine in the blood with the following ordinary differential equations:

$$\frac{d[NH_3]}{dt} = V_{NH3abs} + V_{GLS} - V_{Urea\ for\ Balance} - V_{CPS1} - V_{NH3ex} - V_{GLUL} \quad (1)$$

$$\frac{d[Urea]}{dt} = V_{CPS1} - V_{Urea\ ex} \quad (2)$$

$$\frac{d[Gln]}{dt} = V_{GLUL} - V_{GLS} \quad (3)$$

The overall reaction scheme is shown in **Figure 4.1**. Equations for reaction velocities in Equations 1, 2, and 3 are shown in **Table 4.1**. Carbamoyl phosphate synthetase 1 (CPS1) catalyzes the first committed step in the urea cycle, so this is the only enzyme of the cycle incorporated into the model for simplicity. Other model parameters are shown in **Table 4.2**. Mammalian enzymes from liver tissue were used when the data was available (see Table 4.1). No distinction is made between NH_3 and NH_4^+ in this study unless otherwise noted. N-acetyl-glutamate (NAG) is a CPS1 activator that increases in concentration when more protein is consumed (Morimoto et al. 1990). To model the effects of NAG on CPS1 activity, we interpolated data on CPS1 activity changes from a study that included the effects of changes in hepatic mitochondrial NAG levels due to diet (Morimoto et al. 1990)

(about 11% in this study). The maximally activated activity of CPS1 (Pierson and Brien 1980) was adjusted to reflect the reduced activity at physiological NAG concentrations (McGivan et al. 1976; Caldovic and Tuchman 2003) for all the protein diets and liver conditions used in the study. The adjusted CPS1 values for individuals on the three diets of differing protein content are as follows: 71 g protein per day, 8.05 mmoles/min; 100 g protein per day, 8.47 mmoles/min; 122 g protein per day, 8.78 mmoles/min. Adjustments for liver conditions are described below.

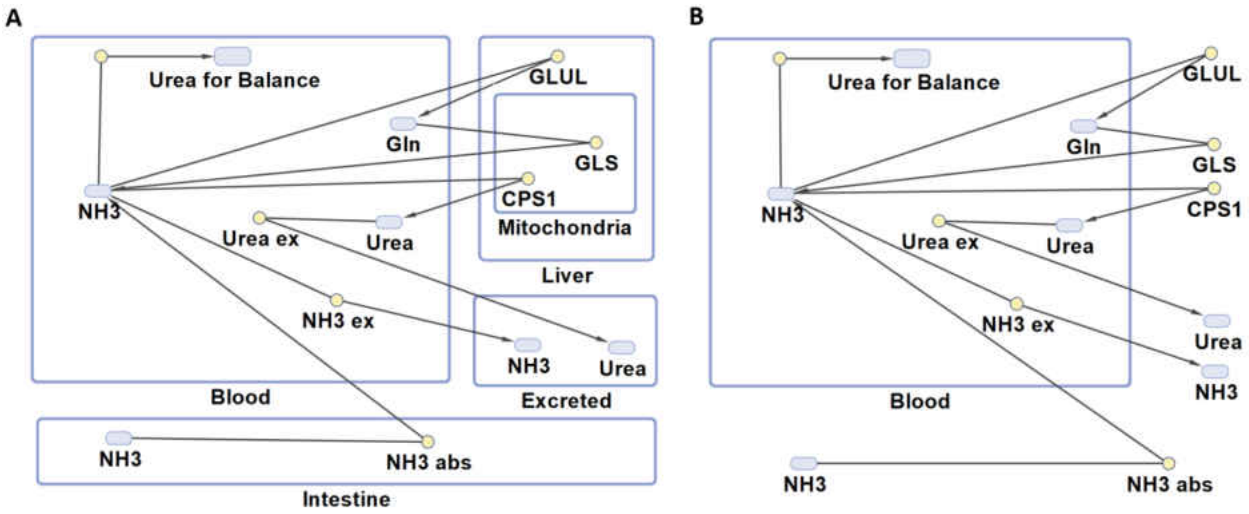

Figure 4.1: Model of nitrogen metabolism and excretion. NH3 in the figure includes both ammonia and ammonium ions. Circles represent reactions (see Table 4.1 for reaction equations) and ovals represent reactants and products. A) Conceptual framework of the *in vivo* physiology simulated including the many tissues and subcellular compartments involved. B) The system is modeled *in silico* considering only the concentrations of metabolites in the blood. Abbreviations are as follows: GLUL, glutamine synthetase; Gln, glutamine; GLS, glutaminase; CPS1, carbamoyl phosphate synthetase 1; Urea ex, urea excreted; NH3 abs, ammonia absorbed; NH3 ex, ammonia excreted.

Table 4.1 Values for reaction velocities scaled to average healthy male liver mass with healthy protein diet

Reaction Velocities	Parameters from the Literature	Organism and Tissue	Parameters Scaled to Liver
$V_{NH3\ abs}$ = 0.492 mmol/min[a]			
$V_{GLS} = (V_{max\ GLS}[Gln])/(K_{m\ Gln} + [Gln])$	$V_{max\ GLS}$ = 91.4 nmole/min per mg (McGivan et al. 1991)	Rat Liver	$V_{max\ GLS}$ = 28.54 mmol/min
	$K_{m\ Gln}$ = 4.0 mM (DeLaBarre et al. 2011)	Human Recombinant	$K_{m\ Gln}$ = 4.0 mM
$V_{CPS1} = (V_{max\ CPS1}[NH_3])/(K_{m\ NH3} + [NH_3])$	$V_{max\ CPS1}$ = 45 nmole/min per mg (Pierson and Brien 1980)	Human Liver	$V_{max\ CPS1}$ = 8.05 mmol/min[ab]
	$K_{m\ NH3}$ = 0.35 mM (Ahuja and Powers-Lee 2008)	Human Recombinant	$K_{m\ NH3}$ = 0.35 mM
$V_{NH3\ ex}$ = 0.004 mmol/min[a]			
$V_{GLUL} = (V_{max\ GLUL}[NH_3])/(K_{m\ NH3}\{1 + ([Gln]/K_{i\ Gln})\} + [NH_3])$	$V_{max\ GLUL}$ = 0.47 µmole/15 min per mg (Tate et al. 1972)	Rat Liver	$V_{max\ GLUL}$ = 12.3 mmol/min[b]
	$K_{m\ NH3}$ = 0.15 mM (Listrom et al. 1997)	Human Recombinant	$K_{m\ NH3}$ = 0.15 mM
	$K_{i\ Gln}$ = 0.6 mM (Wray and Fisher 2005)	*Bacillis subtilis*	$K_{i\ Gln}$ = 0.6 mM
$V_{Urea\ ex}$ = 0.244 mmol/min[a]			

[a]These values change with protein diet. See methods and discussion for details.
[b]These values change with liver condition. See methods and discussion for details.

Table 4.2 Parameter values used in nitrogen metabolism model

Parameter Name	Value	References
Blood Volume	6.59 L[a]	
Average Liver Mass	1561 g	(Molina and DiMaio 2012)
Average Human Mass, Male	88.7 kg	(Fryar et al. 2012)
Time Through Sinusoid	4.3 s	(Schwen et al. 2015)
Initial Urea	5.5 mM	
Initial Ammonia	0 μM	
Initial Glutamine	0 mM	
Recommended Daily Protein	71 g	(2005)

[a]Blood volume is about 5.2 L for a 70 kg individual (Wicker 2015). The value used above was determined by assuming a linear relationship of blood volume with body mass for an individual weighing 88.7 kg. This is the volume of the blood compartment used to calculate metabolite concentrations.

Because all the chemical species under consideration are present in the blood compartment, it is the only compartment where the volume affects simulation results. The other compartments in Figure 4.1 are used to organize the model components for conceptualization. We assume free diffusion across membranes. The volume was determined by assuming a linear relationship between body mass and blood volume and taking 5.2 liters to be the blood volume of a 70 kg male (Wicker 2015). Blood in the model is assumed to be well mixed. Published enzyme activities were scaled up to the average liver size (1561 g, see Table 4.2) by adjusting the units to mmol/(min*1561 g). For example, McGivan et al. (McGivan et al. 1991) report glutaminase activity as 91.4 nmoles per minute per mg protein, which converts to 0.0914 mmoles per minute per g protein. If we assume 20% protein content in the cultured hepatocytes, it takes 5 g of tissue to yield 1 g protein. Adjusting for protein content and multiplying by a liver weight of 1561 g yields an enzyme activity of 28.54 mmoles/min/liver. Because of the slightly higher protein

content of liver tissue compared to isolated hepatocytes (Berry et al. 1991), we used a tissue protein content value of 25% when scaling up parameters from a study that reports enzyme activity from liver tissue. Similar calculations were performed for each of the liver enzymes. Changes in enzyme activity due to diet and liver conditions are summarized in Table 4.3.

Table 4.3 Parameter changes with protein diet and liver condition

Parameter Name	Protein Diet (g per day)	Healthy (mmoles/min)	Early Cirrhosis (mmoles/min)	Late Cirrhosis (mmoles/min)
Urea ex	71	0.244		
	100	0.343		
	122	0.417		
NH3 ex	71	0.004		
	100	0.008	No change with liver condition	
	122	0.012		
NH3 abs	71	0.492		
	100	0.693		
	122	0.845		
$V_{max\ CPS1}$	71	8.05	5.64	5.64
	100	8.47	5.93	5.93
	122	8.78	6.146	6.146
$V_{max\ GLUL}$	No change with diet	12.3	2.46	0.246

The complete urea cycle uses two nitrogen atoms to synthesize each molecule of urea in one turn of the cycle. However, CPS1 incorporates one nitrogen atom per turn of the cycle. A second nitrogen enters into the cycle from aspartate when argininosuccinate synthetase catalyzes the reaction of aspartate with citrulline. To balance the stoichiometry of the reaction series and to simplify the model, a second reaction equal to CPS1 was created with the product not considered in the simulation results ("Urea for Balance" in

Figure 4.1). Therefore, two nitrogen atoms per unit time are used to produce one urea molecule, satisfying the stoichiometry of the overall reaction series for this simplified model. The rate of nitrogen absorption is based upon the daily amount of nitrogen consumed. The mass of protein ingested is adjusted to the molar amount of nitrogen ingested (16% of the mass of protein ingested) and for the 12.7% loss of nitrogen in feces (Tome and Bos 2000). The remaining molar amount of nitrogen is assumed to be absorbed linearly over a period of 24 h. For example, if 71 grams of protein is ingested, 16% of that amount (11.36 grams) is nitrogen. Assuming a 12.7% loss in feces, this leaves 9.92 grams of nitrogen available for absorption. Because there are 14 grams of nitrogen per mole, there are 0.708 moles of nitrogen available for absorption per day, or 0.49 millimoles of nitrogen per minute. The absorption rate was recalculated using the same method for each of the three protein diets modeled. The ranges of the amounts of ammonia and urea excreted in urine over a 24 h period have been reported (Bingham et al. 1988; Bankir et al. 1996), and in the model we assumed the average values to be excreted linearly over the 24 h period. When modeling altered protein intake, the rates of ammonia and urea excretion were adjusted as well. For the high protein diet, we used the upper values of the reference ranges reported for the amounts of ammonia and urea excreted instead of the average values. This was accomplished by assuming daily nitrogen balance and using calculations similar to those used above to equate molar amounts of nitrogen to grams of protein. For example, using the lower values for the reference ranges of daily ammonia and urea excretion and assuming daily nitrogen balance suggest a dietary intake of about 60 g of protein per day. Ammonia and urea excretion rates were scaled to the dietary protein intake by assuming a linear relationship between the two. Excretion rates that were used

in the model are as follows: for the 71 g per day protein diet, 0.004 mmoles ammonia are excreted per minute and 0.244 mmoles urea are excreted per minute; for the 100 g per day protein diet, 0.008 mmoles ammonia are excreted per minute and 0.343 mmoles urea are excreted per minute; and for the 122 g per day protein diet, 0.012 mmoles are ammonia excreted per minute and 0.417 mmoles urea are excreted per minute. Changes with protein diet are summarized in Table 4.3.

We modelled the spatial separation of enzymes by acinus zones by translating spatial separation into temporal separation. The time a red blood cell takes to travel through a sinusoid has been calculated to be 4.3 seconds (Schwen et al. 2015). Assuming a constant rate and equal division of zones, this is about 1.43 s per zone. By using event functions in the SimBiology software, the enzyme activities of CPS1 and glutaminase were turned on for 2.87 s and then off for 1.43 s while GLUL was turned on. This change in enzyme activities combined with the well-mixed assumption approximates the spatial enzyme separation found in the liver because blood is exposed to CPS1 and glutaminase for twice as long and just prior to exposure to GLUL activity before repeating the cycle. This cycling was repeated for 774 seconds during simulations (**Figure 4.2A**), yielding a pattern of peaks and valleys in the simulation results that is a mathematical artifact of modeling spatial separation as temporal separation.

Steady state levels for ammonia were calculated following model simulations for different levels of protein in the diet (healthy, 71 g per day; average, 100 g per day; high, 122 g per day) and liver conditions (healthy, “early cirrhosis”, “late cirrhosis”, and 50% CPS1 activity) for 12 conditions total. Simulation results were exported to Microsoft Excel, and steady state levels were determined from the data points corresponding to the top of

the resulting ammonia concentration curve. Steady state was defined as the concentration when blood ammonia levels were changing less than 0.002% per second.

Several assumptions and simplifications were used in the model. The urea cycle is simplified to the first committed step and an extra CPS1 reaction was added to maintain the stoichiometry of the entire urea cycle ("Urea for Balance" in Figure 4.1). Other molecular species involved in the included reactions that are not included in the model are assumed to be in abundance, blood is assumed to be well-mixed, and we used temporal separation of enzymes to model spatial separation of enzymes in the liver acinus. Furthermore, GLS activity is the same in zones 1 and 2 in the model, but it may be slightly decreased in zone 2 *in vivo*. We recognize that blood is heterogeneous and blood ammonia levels may be concentrated in some compartments compared to others. For example, arterial blood has been shown to have higher ammonia levels than venous blood in dogs with liver disease (Rothuizen and van den Ingh 1982). This difference of concentrations could limit systemic effects of blood ammonia. However, this was not included in the model. We also assume a continuous nutritional supply for simplicity. Furthermore, the model does not account for the activities of transporters but often assumes free passage of small molecules. There is also evidence for positive cooperativity for the binding of some of the species to enzymes in the model. For example, there is evidence of cooperativity for glutamine binding (Hill coefficient of 1.8) in the kinetics of the glutaminase enzyme (Szweda and Atkinson 1989) and possible glutaminase upregulation by a high protein diet (Ewart and Brosnan 1993) that was not included in the model. However, sensitivity analysis suggests that increases in glutaminase and GLUL activity will not have much effect on ammonia steady state levels. Even with these simplifying assumptions, the model results agree relatively well with the

available clinical data. However, the available clinical data is sometimes incomplete, so further validation is not currently possible.

Sensitivity Analysis

To determine the relative effects of altering enzyme kinetic parameters on the steady state blood ammonia levels, each of the parameters was individually increased or decreased by 50% under the normal simulation conditions for a healthy liver and a protein diet of 71 g per day. The simulations were run under these altered conditions and the steady state levels of ammonia were compared to those under normal conditions. Results are reported in **Table 4.4** as percent change.

Table 4.4 Sensitivity analysis for kinetic parameters.

Enzyme	Parameter	Percent Parameter Change	Blood Ammonia Percent Change
CPS1	V_{max}	150%	-35.4
		50%	110.3
	K_m	150%	52.6
		50%	-50.3
Glutaminase	V_{max}	150%	0.6
		50%	-2.9
	K_m	150%	-1.1
		50%	1.1
Glutamine Synthetase	V_{max}	150%	4.6
		50%	-4.6
	K_m	150%	-2.9
		50%	7.4
	K_i	150%	0.6
		50%	-0.6

SH-SY5Y Culture, Differentiation, and Treatment

SH-SY5Y cells were cultured in a 1:1 mixture of DMEM (high glucose) and Ham's F-12 medium. The medium contained 2.44 g/L sodium bicarbonate, 30 mg/L penicillin, 50 mg/L streptomycin, and 10% FBS. The cells were seeded at a concentration of 1,000 cells per well in 96-well plates and treated with retinoic acid (10 μM) for 4 days with the medium changed every two days. Next, ammonium chloride was added to the medium at the indicated concentrations and the cells were incubated for 24 additional hours.

Protein Assay

After 24 h of treatment with ammonium chloride, the cells were washed in PBS and then lysed with RIPA buffer (50 μl per well for three wells per condition). The lysate was pooled in a microcentrifuge tube and the Pierce BCA Protein Assay was performed in triplicate per manufacturer's instructions.

Software and Statistical Methods

The model was built and simulated using the MATLAB R2016a SimBiology software package. Data was analyzed using Microsoft Excel and GraphPad Prism v7.0. For cell culture studies, three independent experiments were performed and data was analyzed with a repeated measures one-way ANOVA with Fisher's *post hoc* test.

Results

Model Results Approximated Physiological Steady State Ammonia Levels

A computational model estimating blood ammonia levels in healthy individuals and those with liver disease was constructed. The model was first simulated using the parameters for a healthy liver and a healthy, moderate protein diet to determine if the resulting steady state levels were consistent with clinical data. The healthy protein diet was taken to be 71 g per day. The reference range for healthy blood ammonia levels was taken to be 11-32 μM (Hawke 2012). Healthy blood urea levels are considered to be 3.6-7.1 mM (Pagana and Pagana 2009). The initial conditions in this study are 0 μM ammonia and 5.5 mM urea. Figure 4.2A shows the results of 774 s of simulation of our model. The steady state ammonia level was 17.5 μM (Figure 4.2A), and the steady state urea level remained around 5.5 mM (Figure 4.2A), both values well within the healthy range.

Enzyme Activity Changes Had Different Effects on Ammonia and Urea Levels

To investigate the relative influences of CPS1 and GLUL on blood ammonia levels, enzyme activities were individually varied stepwise in the model for a healthy individual on a healthy protein diet, and steady state levels of ammonia were determined. The results show an inverse, non-linear relationship between CPS1 activity and ammonia levels (**Figure 4.2B**). The steady state urea level did not show much change under any conditions tested likely because much larger changes are necessary to cause differences in the millimolar concentrations of urea compared to the micromolar concentrations of ammonia. Decreasing GLUL activity had almost no effect on steady state levels of ammonia (Figure 4.2B). However, decreasing GLUL activity does affect the kinetics of ammonia formation.

Eliminating GLUL activity caused the simulation to reach steady state ammonia levels much more quickly. In a healthy individual, the rate of ammonia formation for the first 12.9 seconds of the simulation was 0.55 μmoles per second, but this rate increased to 0.83 μmoles per second when GLUL activity was inactivated (**Figure 4.2C**). A sensitivity analysis (Table 4.4) of the kinetic parameters revealed that, as expected, changes in CPS1 activity have by far the strongest effect on blood ammonia levels of any enzyme in the model.

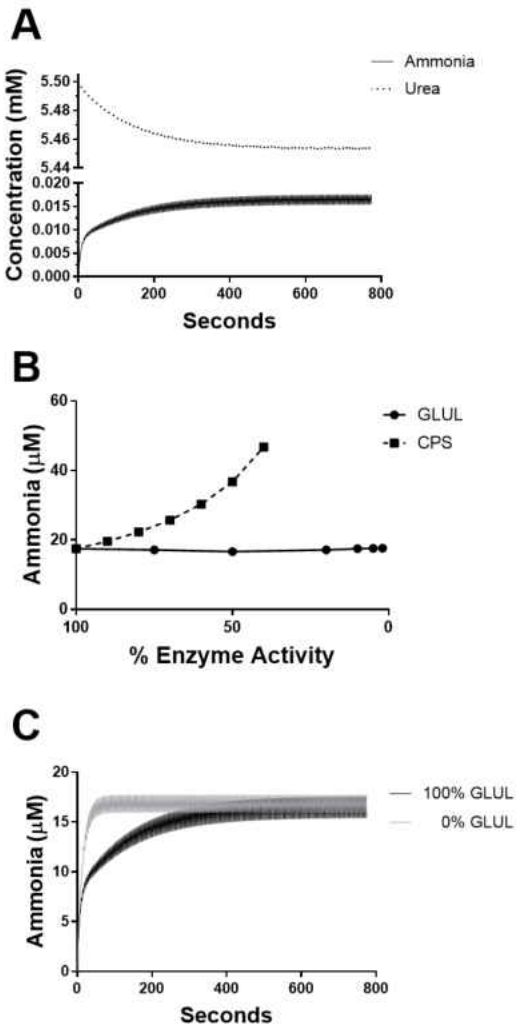

Figure 4.2: Model simulation results for a healthy individual on a healthy protein diet. A) Simulation results for a healthy individual with the recommended daily protein intake. Note the break in the scale of the y-axis. B) Ammonia steady state levels from simulations varying enzyme activities of CPS1 and GLUL. C) Simulation results showing changes in ammonia kinetics with changes in GLUL activity.

Changes in Enzyme Activities Caused by Liver Cirrhosis Affected Blood Ammonia Levels

Liver cirrhosis has been shown to decrease the activities of two of the enzymes in the model, so decreasing the V_{max} values accordingly can create a simple model of liver cirrhosis at different stages. As mentioned above, a healthy, moderate protein diet for a typical adult male is 71 g of protein per day. The average American diet is about 100 g of protein per day, and a high protein diet in our model was taken to be 122 g of protein per day. The amount of protein in the high protein diet was calculated by using the highest values of ammonia and urea excretion in the published reference ranges (Bingham et al. 1988; Bankir et al. 1996) and assuming nitrogen balance (see Methods). To model early stage liver cirrhosis, the V_{max} of CPS1 was reduced to 70% of the normal value and the V_{max} of GLUL was reduced to 20% of the normal value (Gebhardt and Reichen 1994). Late stage liver cirrhosis was modeled by changing the V_{max} values for CPS1 and GLUL to 70% and 2% of the normal values, respectively (Fleming and Wanless 2013). Following simulation, the steady state ammonia and urea levels were determined as described above. The steady state ammonia levels under the various conditions are shown in **Figure 4.3A**. Ammonia levels progressively increased with increased dietary protein intake and with decreased liver function.

Decreased CPS1 Activity Led to Increased Blood Ammonia Levels

Based upon the population frequency of the genetic disorder CPS1 deficiency, there are likely many heterozygous individuals with decreased CPS1 activity (see Discussion). To investigate the consequences of decreased CPS1 activity on blood ammonia and urea levels, the V_{max} for CPS1 was reduced by 50% and different protein levels in the diet were

compared (**Figure 4.3B**). Decreased CPS1 activity (50% below normal, as present in a heterozygous individual with a complete loss of function from one of the two alleles) led to about a 20 μM increase in blood ammonia levels, greater than the levels present in the early or late stage liver cirrhosis models for each of the diets examined.

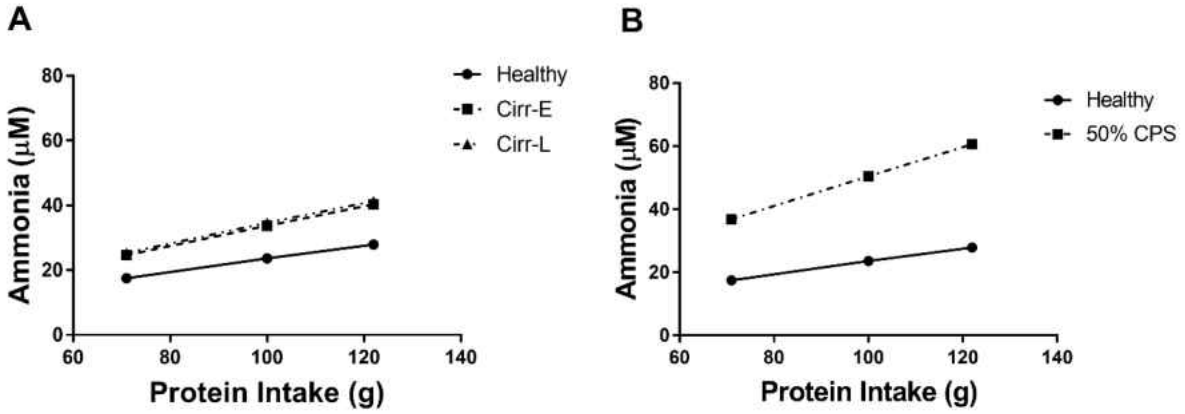

Figure 4.3: Ammonia levels changed with dietary protein levels, progression of liver cirrhosis, and CPS1 activity. A) The steady state ammonia concentration in the blood increases with increased ammonia absorption due to increases in dietary protein (See Table 4.1) and declining liver function. B) When CPS1 activity is reduced by half, steady state ammonia levels are higher than those in a healthy individual. This effect increases with increased ammonia absorption due to increased dietary protein levels (See Table 4.1). Cirr-E, early liver cirrhosis; Cirr-L, late liver cirrhosis.

Ammonium Chloride Treatment Decreased Viability of Differentiated SH-SY5Y Cells

To test whether the increased ammonia levels observed in the simulations could be neurotoxic *in vitro,* we administered ammonia to differentiated human neuroblastoma cells. Retinoic acid-treated SH-SY5Y cells treated with 90 μM ammonium chloride showed 14% decreased viability as measured by protein content from cells attached to the plate

after a PBS wash. Ammonium chloride concentrations of 30 μM or 60 μM showed no statistically significant effect on viability (**Figure 4.4**).

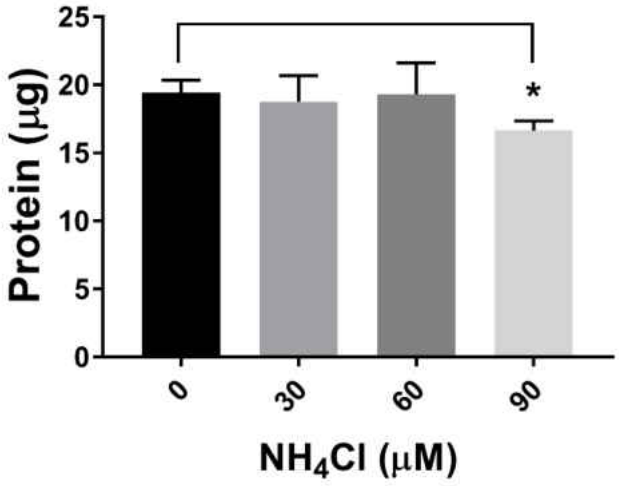

Figure 4.4: Ammonium chloride (90 μM) decreased the viability of retinoic acid-differentiated SH-SY5Y neuroblastoma cells in culture. * indicates that $p = 0.01$ compared to 0 μM NH_4Cl added. Bars represent mean $\pm$ SEM, and $n = 3$ independent experiments.

Discussion

A simple mathematical model of human organismal nitrogen metabolism is presented that uses published parameters for physiological inputs to give physiologically relevant outputs consistent with the available experimental data. While other models exist for ammonia metabolism in humans (Ohno et al. 2008; Drasdo et al. 2014; Schliess et al. 2014), this is the first to model the effects of altered levels of dietary protein intake on blood ammonia levels. Increased protein intake led to increased blood ammonia levels

across all conditions. However, blood ammonia levels remained in the healthy range when simulating a healthy liver. The simulation results showed that low GLUL activity can lead to more rapid changes in blood ammonia levels. The model highlights the effects of diet on ammonia levels in disease conditions. Our results indicate that increased protein intake likely causes blood ammonia to rise above healthy levels in some patients with cirrhosis.

Altering Dietary Protein Consumption in Cirrhosis Patients

Because relative energy expenditure per kg body mass is increased in some cirrhotic patients (Merli et al. 1985), some sources recommend a high protein diet (1.8 g/kg per day) for these patients to maintain muscle mass if they do not already have HE (Nielsen et al. 1995). Others suggest a normal, moderate intake of 0.8 g to 1.0 g of protein per kg per day (Riordan and Williams 1997). Data has shown that high protein intake exacerbated encephalopathy in 35% of patients with cirrhosis (Seymour and Whelan 1999). Reduced protein intake was first shown to protect from encephalopathy in cirrhotic patients in 1952 (Phillips et al. 1952). In 2004, a study was performed with cirrhosis patients where protein was completely removed from the diet for three days and then slowly increased over 12 days back to the normal level (Córdoba et al. 2004). At the end of the study, blood ammonia levels were non-significantly 17% lower in the patients with the restricted protein diet compared to patients on a normal protein diet of 1.2 g/kg per day, roughly equivalent to the average American protein diet in this model. However, the methods used in that study have been critiqued and questioned (Nguyen and Morgan 2014). A larger patient group size and earlier measurements of blood ammonia levels would help to clarify if blood ammonia levels are indeed decreased by a low protein diet. If the findings of no

significant effects do prove to be robust and highly reproducible, this leaves the possibility that a high protein diet may play a role in the development of HE, but a low protein diet is not normally helpful in its resolution. Even though several studies have suggested that restricting dietary protein intake below the recommended amount for a healthy individual may not be therapeutic for the roughly 60% of cirrhosis patients who suffer from malnutrition, other studies over the past 65 years on HE patients who can maintain a proper energy balance have consistently shown benefits of protein restriction (Nguyen and Morgan 2014). We acknowledge the large heterogeneity in patient responses to changes in the level of dietary protein (Gheorghe et al. 2005) and suggest that monitoring the cirrhosis patient's energy balance will help determine the proper dietary protein level for that individual.

Previous research has shown that there was an 80% reduction in GLUL activity and a 30% reduction in CPS1 activity in a rat model of liver cirrhosis (Gebhardt and Reichen 1994). As the disease progressed, GLUL activity dropped even further (Fleming and Wanless 2013). Results from adjusting the model to these parameters suggest that ammonia levels will increase as liver cirrhosis develops. Furthermore, a high protein diet may exacerbate these effects. We recognize that liver cirrhosis is a complex disease with many changes besides altered CPS1 and GLUL activity, so this model is a simplified representation of liver cirrhosis. Using an upper limit of 32 μM for the reference range of healthy blood ammonia levels, the model indicates that a high protein diet with early or late cirrhosis will result in blood ammonia levels that are at least 20% higher (40.3 μM and 41.3 μM, respectively) than the upper limit for the healthy range (32 μM). These elevations may contribute to HE. The model suggests that controlling protein intake could be one

method to slightly reduce the likelihood of developing HE in some patients with liver cirrhosis.

Plasma Ammonia Levels Likely Rise in Healthy Individuals After a Meal

Our model predicts that blood ammonia levels will rise slightly by consumption of a high protein diet. Surprisingly, we could not find many studies in the literature examining dietary-induced changes in blood ammonia levels in healthy humans. One study found increased blood ammonia levels in women following consumption of a test drink containing whey protein. The ammonia level peaked at a value 20% higher than the initial level at 90 minutes after consumption (Chungchunlam et al. 2015). Another study found increasing breath ammonia levels after a high protein challenge; ammonia levels plateaued roughly 5 hours after the dietary challenge (Spacek, M.L. Mudalel, et al. 2015). However, breath ammonia levels do not always correlate well with blood ammonia levels (Spacek, M. Mudalel, et al. 2015). A further study using Huntington's disease patients who were put on a high (26.3%) protein diet did not find any association between the high protein diet and blood ammonia levels (Chen et al. 2015).

In studies with mice placed on a high protein diet ammonia levels increased from 210 μM to 245 μM at night when the mice were active and feeding. Likewise, a high fat/low protein diet decreased ammonia levels from 170 μM to 130 μM when measured at night (Nohara et al. 2015). When rats were switched from a 20% protein diet to a 58% protein diet, colonic venous ammonia maximally increased from 100 μM to 340 μM two days after the switch in diet, which dropped to 170 μM after a week on the diet (Mouillé et al. 2004). Another study using rats trained on a 6% protein diet showed that blood ammonia levels

increased from 60 μM to 120 μM when they were given a 44% protein meal, and ammonia levels were maintained at that elevated level for at least 24 hours (Semon et al. 1988). There is also evidence of increases in blood ammonia levels in pigs after a protein meal (Welters et al. 1999; Liu et al. 2015). The limited data above suggest that systemic ammonia levels likely increase slightly following a meal, especially if the meal is high in protein, but more experiments should be performed to verify these initial findings and to determine the extent of brain ammonia level changes under the same conditions. In addition, more detailed studies using human subjects would help to better characterize the time dependency of elevated ammonia levels as well as to discern differential effects in patients with liver disease.

Variability in the Correlation between Blood Ammonia Levels and the Severity of HE

While there is a correlation between blood ammonia levels and HE severity (Ong et al. 2003), the exact blood concentration that leads to impairment may be different for individuals based on their specific nitrogen balance and the release of other neurotoxic factors from the liver. Some patients with hyperammonemia present with 100-200 μM blood ammonia levels and remain asymptomatic, while others such as infants with GLUL deficiency have severe encephalopathy with blood ammonia levels fluctuating between 100 and 150 μM (Häberle 2013). However, decreased blood glutamine levels may also contribute to the encephalopathy in GLUL deficiency. Maintained blood ammonia levels over 300 μM are considered severe and invariably lead to encephalopathy. However, some newborns have been shown to have no lasting impairment by temporary blood ammonia concentrations up to 2 mM for a day or two (Whitelaw et al. 2001). In contrast with this

data, blood ammonia levels (< 50 μM) that border the healthy range have also been linked to HE (Ong et al. 2003). These variabilities do not affect the validity of the model because the model represents the blood ammonia level changes for an average person.

To investigate the roles of CPS1 and GLUL in ammonia metabolism, enzyme activities were varied in the model with otherwise healthy parameters (Figure 4.2B, Table 4.4). CPS1 activity levels relevant to the model had a non-linear effect on ammonia concentrations. GLUL activity, however, had very little effect on ammonia steady state levels. Simulating early liver cirrhosis (70% CPS1, 20% GLUL) resulted in an average ammonia increase of 43% across all diets. Simulating late liver cirrhosis (70% CPS1, 2% GLUL) resulted in an average ammonia increase of 47%. GLUL activity appears to slow changes in blood ammonia levels. Since muscles may help metabolize some ammonia (Dejong et al. 1992), slowing the rate of change may give the body time to adapt to the larger ammonia levels. These results are consistent with the known role of CPS1 together with the rest of the urea cycle to be a low affinity, high capacity system for removing ammonia, while GLUL is a high affinity, low capacity enzyme for removing ammonia (Häussinger 1986).

Ammonia Levels in Individuals Deficient in GLUL

Human subjects with decreased GLUL activity have been shown to have blood ammonia levels of 100-150 μM (Häberle 2013), while mice with liver-specific GLUL knockout showed a blood ammonia level of roughly 150 μM (Qvartskhava et al. 2015). Our model, under otherwise normal healthy conditions, shows no change in ammonia steady state levels when GLUL is reduced (Figure 4.2B). This is a limitation of the study possibly

due to the enzyme kinetics data available, but it could also be due to the way we have modeled the hepatic acinus, the functional unit of the liver. The ratio of CPS1 to GLUL activity or the amount of time that ammonia is associated with CPS1 activity compared to GLUL activity may be too high in our model, resulting in deviations from *in vivo* results. This suggests that some of the changes in ammonia levels observed in our model may be too conservative.

Ammonia Levels in Individuals Deficient in CPS1

CPS1 deficiency is a rare autosomal recessive genetic disorder that results in very little CPS1 activity (McReynolds et al. 1981). Individuals experience extreme hyperammonemia and the many detrimental effects that come with it (Klaus et al. 2009). The prevalence of CPS1 deficiency is about 1 in 800,000 (Nagata et al. 1991). Assuming strict Mendelian inheritance, if a mother and father each are heterozygous and have decreased CPS1 activity, there is a 1 in 4 chance that their children will have CPS1 deficiency. Working backwards from this assumption, the odds that both parents are heterozygous are 1 in 200,000. The odds that one parent is heterozygous is roughly 1 in 447. Therefore, heterozygosity for disease-causing CPS1 mutations is almost as prevalent as liver cirrhosis. However, regulatory effects may partially compensate for CPS1 heterogeneity, a scenario not explored is this study. The model predicts that individuals with CPS1 activity 50% of normal levels (currently thought to have no detrimental effect) will have high blood ammonia levels. Many people may be unaware that they carry a mutation in one CPS1 allele that likely results in higher than normal levels of blood

ammonia. Lifelong exposure to high levels of blood ammonia may have unknown, deleterious effects on neural function.

Relatively Low (90 μM) Brain Ammonia Levels May Affect Neural Cell Viability or Function

It is hypothesized that the increased levels of ammonia in liver disease interfere with the glutamine-glutamate balance involved in neurotransmission (Butterworth 2014), which can lead to increased production of reactive oxygen and nitrogen species (Bobermin et al. 2015). Increased brain ammonia levels also block potassium uptake in astrocytes, which causes increased potassium uptake in neurons that compromises inhibitory neurotransmission in the cortex, leading to seizure (Rangroo Thrane et al. 2013).

Cell culture experiments using retinoic acid-differentiated SH-SY5Y cells revealed that viability was decreased by relatively low concentrations (90 μM) of ammonium chloride. The ability of such a low concentration of brain ammonia to cause toxicity was surprising given that most cells (Schneider et al. 1996) including rodent primary cortical and cerebellar granule cells and undifferentiated human SH-SY5Y cells require low (1-10) millimolar concentrations of ammonium chloride before toxicity is observed (Bobermin et al. 2015). Ammonia has been reported to be slightly more toxic to neuroblastoma cells than to primary neurons (Haghighat et al. 2000), partially accounting for the lower toxicity threshold. Furthermore, the retinoic acid-mediated differentiation procedure we used likely sensitized the cells to ammonia toxicity as it is known to increase reactive oxygen species production (Kunzler et al. 2016).

The ability of uncharged NH_3 to cross the blood brain barrier and the limited ability of the charged, protonated form NH_4^+ to cross the barrier combined with the difference in

pH between the brain and the blood allows higher total ammonia levels to accumulate in the brain (Auron and Brophy 2012). The model predicts serum (pH ~ 7.4) levels of ammonia/ammonium in healthy individuals will be 17.5 μM; since the brain has a pH of about 7.0 (Shi et al. 2014; Ren et al. 2015), applying the Henderson-Hasselbalch equation implies a total ammonia/ammonium concentration in the brain of 44 μM for a healthy individual on a healthy protein diet, more than twice the blood ammonia/ammonium concentration. The following diet combinations and liver conditions are predicted from the model results to have brain ammonia/ammonium concentrations of > 90 μM: high protein diet/early cirrhosis (101 μM), high protein diet/late cirrhosis (104 μM), healthy protein diet/decreased CPS1 activity (92 μM), average protein diet/decreased CPS1 activity (127 μM), and high protein diet/decreased CPS1 activity (152 μM). Combining the experimental data with the computational results suggests that many of the diet and liver condition combinations could negatively affect neuronal function. Mouse studies showing that high protein diets are associated with decreased lifespan (Solon-Biet et al. 2014) should stimulate further research into the mechanisms involved. Adhering to the recommended protein diet may help cirrhosis patients and CPS1 heterozygotes avoid high blood and brain ammonia levels and any associated cognitive problems.

Conclusions

The following testable hypotheses have been generated using this model: 1) Increasing dietary protein consumption increases blood ammonia levels in healthy individuals. 2) A low protein diet is beneficial for liver cirrhosis patients who have a normal energy balance. 3) Heterozygosity for CPS1 complete loss of function mutations

leads to elevated blood ammonia levels. 4) Chronic but low-level hyperammonemia has negative effects on neurons and astrocytes such as sensitizing them to further toxic insults. 5) Increased blood ammonia levels contribute to the decreased lifespan of mice on a high protein diet (Solon-Biet et al. 2014).

This model describes physiological and pathophysiological human nitrogen metabolism in blood and liver using published parameters. It suggests that the diet and the level of progression of liver cirrhosis contribute to blood ammonia levels, and experimental results suggest that these blood ammonia levels could affect neural functioning. Since high blood ammonia levels are associated with diseases such as HE, the model can be used to predict the conditions in which HE may develop. Furthermore, the model predicts that a 50% reduction in CPS1 activity, an activity level likely present in thousands of individuals worldwide, can lead to high blood ammonia levels. Limiting protein intake may be one effective way for some of these individuals to decrease blood ammonia levels and possible associated pathologies.

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CHAPTER 5

AMINO ACID CATABOLISM IN ALZHEIMER'S DISEASE BRAIN:

FRIEND OR FOE?

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Abstract

There is a dire need to discover new pathways to target for Alzheimer's disease (AD) drug development. The decreased neuronal glucose metabolism that occurs in AD brain could play a central role in disease progression. Little is known about the compensatory neuronal changes that occur to attempt to maintain energy homeostasis. In this review using the PubMed literature database, we summarize evidence that amino acid oxidation can temporarily compensate for the decreased glucose metabolism, but eventually altered amino acid and amino acid catabolite levels likely lead to toxicities contributing to AD progression. Because amino acids are involved in so many cellular metabolic and signaling pathways, the effects of altered amino acid metabolism on AD are far-reaching. Possible effects resulting from changes in the levels of several important amino acids are discussed. Urea cycle function may be induced in endothelial cells of AD patient brains, possibly to remove excess ammonia produced from increased amino acid catabolism. Studying AD from a metabolic perspective provides new insights into AD pathogenesis and may lead to the discovery of dietary metabolite supplements that can partially compensate for alterations of enzymatic function to delay AD or alleviate some of the suffering caused by the disease.

Keywords

Amino Acids, Alzheimer's Disease, Urea Cycle, Metabolism, Supplementation

Background

There are currently about 24 million cases of Alzheimer's disease (AD) worldwide, and that number is expected to continue to increase for at least the next few decades as better treatments for other diseases such as heart disease and cancer extend average human longevity (Reitz et al. 2011). In addition to human suffering, Alzheimer's disease and other dementias cost the United States about $172 billion in 2010 (2010). To date, most research has focused on the amyloid cascade hypothesis that emphasizes the role of amyloid-β protein aggregation in the pathogenesis of AD. However, growing evidence suggests that the amyloid cascade hypothesis does not encapsulate the complex symptomology of AD (Herrup 2015). Two decades of researching the amyloid cascade hypothesis have not yielded the treatments that were predicted in the early 1990s. The other major histological hallmark of AD in addition to amyloid plaques is the neurofibrillary tau tangle pathology present in neurons (Herrup 2015). It is possible that tau-based therapies will not fare better in clinical trials than amyloid-based therapies. Another promising alternative is to view Alzheimer's disease as a metabolic disease in attempt to shed novel insight into its etiology. In this regard, it is known that neurons in AD brain show large deficits in glucose metabolism, so alternative energy sources may help to prevent the neuronal death characteristic of the disease. Treating AD as a metabolic disorder would lead to further research into dietary supplementation of metabolites and enzyme cofactors that show depletion in AD brain as possible treatments. Neurons lack the

enzymes for beta-oxidation of fatty acids, but other possible neuronal energy sources include amino acids, ketone bodies, citric acid cycle intermediates, pyruvate, and lactate. Because many recent metabolomics investigations have shown large changes in the levels of several amino acids in AD brain and plasma, it is important to consider whether changes in amino acid metabolism are a driving force for AD progression.

Amino acids in the form of proteins are a large part of the human diet. The recommended daily allowance of protein is 0.8 grams per kilogram body mass (2005); in an average adult this amounts to roughly 71 grams of protein per day. This high protein consumption dictates that amino acids will be present at levels that far exceed their requirements as the building blocks for protein synthesis, and that much of the protein consumed will be broken down for energy generation. Processing these amino acids for energy generation requires the disposal of nitrogenous waste, a process carried out mostly in the liver and small intestine by the urea cycle. Disrupted amino acid and nitrogen metabolism is associated with neurological defects and in some cases dementia (Wiesinger 2001; Gropman et al. 2007; Coloma and Prieto-Gonzalez 2011; Esposito et al. 2013; P. Liu et al. 2014). In addition to these primary routes of amino acid usage, amino acids and their metabolic derivatives are also involved to a lesser extent in cell signaling and in many diverse metabolic pathways.

Amino Acid Metabolism in the AD Brain

Many studies have shown altered amino acid levels in serum and brain in AD patients or in AD model mice, but whether these changes contribute to disease pathogenesis is not yet known. Because glutamate is an excitatory neurotransmitter and

two of its metabolites, gamma-aminobutyric acid (GABA) and glycine, are inhibitory neurotransmitters (Esposito et al. 2013), changes in glutamate metabolism in AD brain could greatly affect neural functioning. GABA can also be synthesized from arginine in astrocytes and increased GABA levels synthesized through this pathway have been shown to play a role in cognitive dysfunction in an AD mouse model (Jo et al. 2014). Changes in the levels of enzymes involved in amino acid metabolism have also been observed in AD brain (Jęśko et al. 2016), further suggesting a role for metabolic dysregulation in AD pathogenesis. For example, alterations in the levels of glutamine synthetase and urea cycle enzymes and intermediates have been observed in AD brain. This is of concern because nitrogenous waste in the form of ammonia from amino acid catabolism has adverse effects on neural cells if not properly cleared from the brain.

A healthy individual is able to process the excess amino acids consumed into other useful metabolites or oxidize them for energy production. To use them as fuel, the carbon skeletons are oxidized in the citric acid cycle to produce carbon dioxide, and the excess nitrogen is disposed of as the relatively non-toxic nitrogenous waste product urea. However, when neurons cannot catabolize glucose efficiently such as during AD, they likely become partly reliant upon amino acid oxidation for energy. If neuronal amino acids become depleted or if the machinery used to metabolize amino acids becomes dysregulated, the neurons may die, contributing to disease progression. However, even if amino acid oxidation is able to maintain neuronal energy levels, the increased amounts of ammonia released during amino acid catabolism could lead to neuronal cell death because a complete urea cycle that is needed to detoxify ammonia is not present in neurons.

Instead, astrocytes contain the glutamine synthetase enzyme to sequester ammonia into glutamine, which can be released from the brain.

Amino Acid Level Changes Examined in Control, MCI, and AD Populations

Identifying amino acid changes in patients with mild cognitive impairment (MCI) is important as these changes may be upstream changes leading to the onset of AD, and the downstream changes that occur during AD may be a result of the body adapting to the insults that occurred during MCI. In one report, a metabolomics analysis was performed on the cerebralspinal fluid (CSF) of patients with mild cognitive impairment (MCI), AD, or healthy aged-matched controls. Results showed that the metabolites dimethylarginine, arginine, valine, proline, serine, histidine, choline, creatine, carnitine, and suberylglycine were possible disease progression biomarkers (Ibáñez et al. 2012). Another group studying potential CSF biomarkers for AD concluded that changes in methionine, tryptophan, tyrosine, and purine metabolism pathways occurred in both MCI and AD subjects. Methionine levels increased in MCI while tryptophan levels decreased (Kaddurah-Daouk et al. 2013). Levels of the tripeptide glutathione also decreased in AD. Another study found increased cysteine levels in CSF from AD subjects (Czech et al. 2012) while another identified altered tryptophan and phenylalanine levels in plasma from both MCI and AD subjects compared to controls; tryptophan levels were also distinct when comparing MCI to AD subjects (Y. Liu et al. 2014). A further metabolomics study of plasma found altered arginine metabolism and polyamine metabolism in MCI and AD subjects (Graham et al. 2015). Another study found that glycine and valine levels were altered in AD plasma (Klavins et al. 2015), but the authors warned that plasma amino acid levels

show large variability depending upon the amount of fasting the subjects had undergone prior to donating blood (Mathew et al. 2014) and that phospholipids may be more reliable plasma biomarkers.

A comprehensive metabolomics study of both plasma and CSF from control, MCI, and AD subjects found that tryptophan and arginine metabolism were altered in both CSF and plasma from MCI subjects (Trushina et al. 2013). Lysine metabolism was decreased in the CSF, but not plasma, from the MCI subjects. This study also found increased methionine levels in the CSF of MCI subjects. Methionine, histidine, and lysine levels were increased in AD plasma. The pathways affected in AD in both CSF and plasma included beta-alanine, aspartate and asparagine, alanine, cysteine, methionine, methionine-cysteine-glutamate, and arginine and lysine metabolism. Phenylalanine, lysine, and leucine were three of six metabolites in plasma that could be used to discriminate between the MCI subjects and controls (Trushina et al. 2013). A salivary metabolomics analysis found that taurine and several dipeptides including Ser-Ser, Phe-Pro, and Arg-Leu were decreased in abundance in MCI patients (Zheng et al. 2012). From the summation of these results one can discern that there are many alterations in amino acid metabolism in MCI and AD patients, but the results are not very consistent from study to study likely due to the different methodologies and instrumentation used.

An Overview of Select Amino Acids by Class

In AD and MCI patients, some amino acids showed increased levels while most showed decreased levels, especially in the brain, consistent with their oxidation as a neuronal energy source. This information is summarized in **Table 5.1**. There are several

possible connections described in detail below through which disruption of normal amino acid metabolism may lead to pathogenicity.

Table 5.1: Amino acids of interest in neurodegeneration

Amino Acids of Interest	Tissue	Increase or Decrease in AD	Points of Interest	Effects of Addition	DataWarrior Drug Score*
Leucine			Led to persistent mTOR activation (Lynch and Adams 2014); Metabolites led to metabolic dysfunction (Amaral et al. 2010; Lynch and Adams 2014; Wisniewski et al. 2016)	Metabolite led to mitochondrial dysfunction in rat neurons (Amaral et al. 2010); BCAAs increased mean lifespan in male mice (D'Antona et al. 2010)	0.592
Valine	Plasma (Gonzalez-Dominguez et al. 2015)	Decrease (Gonzalez-Dominguez et al. 2015)	Led to persistent mTOR activation (Lynch and Adams 2014); Metabolites led to metabolic dysfunction (Lynch and Adams 2014)	BCAAs increased mean lifespan in male mice (D'Antona et al. 2010)	0.559
Isoleucine			Led to persistent mTOR activation (Lynch and Adams 2014); Metabolites led to metabolic dysfunction (Lynch and Adams 2014)	BCAAs increased mean lifespan in male mice (D'Antona et al. 2010)	0.684
Phenylalanine	Serum (Gonzalez-Dominguez et al. 2015); Brain (Nilsen et al. 2014; Xu et al. 2016)	Decrease (Gonzalez-Dominguez et al. 2015); Increase (Nilsen et al. 2014; Xu et al. 2016)	Metabolized in absence of tyrosine (Fernstrom and Fernstrom 2007)		0.579
Tryptophan	Serum (Gonzalez-Dominguez et al. 2015); Brain (Xu et al. 2016)	Decrease (Gonzalez-Dominguez et al. 2015); Increase (Xu et al. 2016)	Metabolites led to metabolic dysfunction (Bonda et al. 2011)	Metabolite increases nitric oxide synthetase in cell culture (Pérez-Severiano et al. 1998)	0.661

Tyrosine	Serum (Gonzalez-Dominguez et al. 2015)	Decrease (Gonzalez-Dominguez et al. 2015)	Decreases could disrupt catecholamine production	Improved memory and cognitive function in humans (van de Rest et al. 2013)	0.584
Glutamine	Serum (González-Domínguez et al. 2015); Brain (Gueli and Taibi 2013)	Decrease (González-Domínguez et al. 2015); Increase (Gueli and Taibi 2013)			0.573
Aspartic Acid	Brain (Gueli and Taibi 2013; Xu et al. 2016); Serum (Gonzalez-Dominguez et al. 2015)	Decrease (Gueli and Taibi 2013; Gonzalez-Dominguez et al. 2015; Xu et al. 2016)			0.593
Glutamic Acid	Serum (G. Wang et al. 2014); Brain (Gueli and Taibi 2013; Xu et al. 2016)	Increase (Xu et al. 2016); Decrease (Gueli and Taibi 2013; G. Wang et al. 2014)	Excitotoxicity led to neuronal death (Mattson et al. 1992; Hynd et al. 2004)		0.531
Lysine	Brain (Xu et al. 2016)	Decrease (Xu et al. 2016)			0.499
Histidine	Serum (Gonzalez-Dominguez et al. 2015)	Decrease (Gonzalez-Dominguez et al. 2015)			0.835
Cysteine	Serum (González-Domínguez et al. 2014; Hu et al. 2016); Brain (Hu et al. 2016; Xu et al. 2016); CSF (Czech et al. 2012)	Decrease (Hu et al. 2016); Increase (Czech et al. 2012; González-Domínguez et al. 2014; Xu et al. 2016)	Involved in glutathione synthesis	Decrease in mTOR activity in rats (Gomez et al. 2015)	0.493

Methionine	Serum (González-Domínguez et al. 2014)	Decrease (González-Domínguez et al. 2014)	Increased amyloid-beta and p-tau in mice (Tapia-Rojas et al. 2015)	0.578

*Structures were drawn using DataWarrior software using their most prevalent charge states at pH 7.4. A higher Drug Score value indicates a better drug candidate.

Branched Chain Amino Acids, mTOR, and AD

The branched chain amino acids (BCAAs) include leucine, valine, and isoleucine. BCAAs compete with the aromatic amino acids phenylalanine, tyrosine, and tryptophan for entry into the brain. Therefore, altering plasma BCAA levels can affect the levels of the neurotransmitters serotonin, dopamine, epinephrine, and norepinephrine in the brain (Fernstrom 2005). Unlike most amino acids which are metabolized to a large extent by first pass hepatic metabolism, BCAAs are not metabolized there to a large degree, so their concentration in blood often directly reflects the level of dietary consumption. Protein restriction has been shown to decrease tau hyperphosphorylation and increase cognition in an AD mouse model (Parrella et al. 2013). A BCAA-restricted diet has been shown to induce similar protective metabolic effects on peripheral glucose and insulin levels as a protein restricted diet (Fontana et al. 2016). However, whether the neuroprotective effects of protein restriction are mediated by decreased BCAA levels is not yet known because methionine restriction can also induce protective metabolic effects (Sanchez-Roman and Barja 2013).

Some researchers suspect a link between increased BCAA levels and AD pathogenesis (Morabito et al. 2014). Increasing BCAA levels through dietary supplementation in rats led to a decrease in neural growth factor (NGF) in the hippocampus (Scaini et al. 2013), a part of the brain involved in memory formation and

known to shrink due to neuronal loss in AD patients. Administration of the leucine metabolite α-ketoisocaproic acid also decreased NGF as well as brain-derived neurotrophic factor (BDNF) (Wisniewski et al. 2016). However, the role of BCAAs in AD is not clear-cut. The level of one of the BCAAs, valine, was found to be decreased in the plasma of AD patients (Gonzalez-Dominguez et al. 2015). Furthermore, there are several studies that link increased levels of BCAAs to indicators of increased health such as increased muscle protein synthesis (Rennie et al. 2006), mitochondrial biogenesis (D'Antona et al. 2010), and mTOR signaling (Tokunaga et al. 2004). A beneficial role of mTOR signaling in AD has been hypothesized due to the fact that insulin is neuroprotective (Felice 2013; Cai et al. 2015), and insulin can activate mTOR kinase through PI3K (Um et al. 2006; Vergès and Cariou 2015). mTOR activity has also been found to be neuroprotective under other experimental conditions. For example, increased mTOR activation was associated with decreased Aβ pathology in brains from the 5XFAD mouse model (Avrahami et al. 2013). However, these effects may be related to the particular model or to the length of time of mTOR activation. In the short term, it appears mTOR activation can lead to improved insulin secretion (Rachdi et al. 2008), whereas chronic mTORC1 activation may lead to exhaustion of β-cells in the pancreas (Shigeyama et al. 2008), decreasing the levels of neuroprotective insulin. Somewhat contrary to the findings of mTOR being neuroprotective is the finding that increased mTOR activation is frequently found in the brains of AD model mice (Caccamo et al. 2011) and human AD patients (Li et al. 2005). Increased amyloid-beta levels lead to increased mTOR activation which increases protein translation to increase levels of tau protein, the main component of the neurofibrillary tangles pathologically found in AD neurons (Jahrling and Laberge 2015). The increased

rate of translation induced by mTOR activation may be partially responsible for the decreased levels of amino acids measured in AD brain. Increased mTOR activity can also stimulate mitochondrial electron transport chain activity (Schieke et al. 2006), perhaps leading to increased mitochondrial catabolism of amino acids. The increased mTOR activity also decreases the rate of autophagy, leading to the build-up of toxic amyloid-beta peptides. Consistent with these findings, treatment of PDAPP or 3xTg-AD mice with rapamycin, an mTOR inhibitor, reduced amyloid-beta and tau levels and restored cognitive function (Caccamo et al. 2010; Spilman et al. 2010). These data suggest that diets containing low protein levels or low levels of the potent mTOR activators leucine and arginine may prove beneficial for AD patients (Hara et al. 1998), although decreasing the levels of all three BCAAs together was more potent than decreasing only leucine levels on enhancing peripheral metabolism in mice (Fontana et al. 2016).

Since a portion of the protective effects of mTOR inhibition by rapamycin treatment in AD model systems is likely caused through a decreased rate of translation, other therapies which decrease the rate of translation in the hippocampus may also be therapeutic. With this in mind, decreased or unbalanced amino acid levels have also been shown to decrease the rate of translation through the general control nonderepressible 2 (GCN2)-eIF2α kinase pathway. GCN2 kinase senses uncharged tRNAs and then phosphorylates the translation initiation factor eIF2α to slow the rate of translation. Many amino acids, for example BCAAs and aromatic amino acids, share the same amino acid transporter for transport across the blood brain barrier. Therefore, supplementation with high levels of one particular amino acid may competitively decrease the rate of transport of others into the brain to decrease the levels of one or more of these other amino acids. This

could create imbalanced amino acid levels in the brain to activate GCN2 and inhibit mTOR, decreasing global translation rates and increasing autophagy to protect AD brains. Not all mRNA transcripts show decreased translation under amino acid limitation. Some transcripts, such as ATF4, show increased translation to mediate protective compensatory responses. ATF4 is a transcription factor involved in the ER stress response that has been shown to be upregulated during five conditions that extended mouse longevity (Li et al. 2014).

There are two prevalent hypotheses linking BCAAs to metabolic disease (Lynch and Adams 2014). First, BCAAs, especially leucine, may directly lead to persistent activation of mTORC1. Second, hyper-activation of BCAA catabolism can lead to increased BCAA metabolites which lead to metabolic dysfunction. For example, adding the α-keto acid catabolite of leucine, α-ketoisocaproic acid, to rat neurons led to mitochondrial dysfunction (Amaral et al. 2010). The conflicting results of studies attempting to find correlations between BCAA levels and disease suggest a complex role for BCAA levels in metabolism that may vary depending on the model organism, disease state, and the length of time of elevated BCAA and BCAA catabolite levels.

Few studies have examined the levels of BCAAs specifically in postmortem AD brain, but it appears increasing certain BCAAs may be beneficial for the aging brain in specific model systems. A diet high in BCAAs has even been shown to increase the mean lifespan of male mice (D'Antona et al. 2010). Research into the role of BCAAs in AD is far from complete. For example, there has been little study of the effects of isoleucine or valine supplementation in AD patients or animal models. Since isoleucine and valine do not stimulate mTOR activity as potently as leucine does (Xu et al. 1998) and are not broken

down into neurotoxic α-ketoisocaproic acid, supplementation with these amino acids could provide energy for the brain without activating potentially pathogenic signaling pathways. Given the links between BCAAs, mTOR signaling, aging, and neurodegeneration, further research will likely clarify these complex interactions. Furthermore, many of the same correlations observed for BCAAs in insulin resistant individuals have also been observed for aromatic amino acids, highlighting a complementary role for both of these classes of amino acids in metabolism and disease (Newgard 2012).

Aromatic Amino Acids

Several studies have found that the levels of aromatic amino acids are frequently altered in AD serum or brain. One research group found a decrease in all three aromatic amino acids in the serum of AD patients (Gonzalez-Dominguez et al. 2015), while others reported an increase in both phenylalanine and tryptophan in the brains of AD patients (Xu et al. 2016). Researchers using AD model rats found an increase in phenylalanine in different regions of the brain (Nilsen et al. 2014). Tryptophan has two different neural fates; it can be metabolized into serotonin or it can enter the kynurenine pathway (KP) where it is degraded to α-amino-β-carboxymuconate-ε-semialdehyde (ACMS) which can either be metabolized into quinolinic acid for NAD synthesis or into 2-aminomuconate for entry into the TCA cycle. Serotonin plays a role in learning and cognition (Geldenhuys and Van Der Schyf 2011), but enzymes involved in the KP are upregulated in AD (Bonda et al. 2011). There is evidence that quinolinic acid (QUIN) and other metabolic intermediates in the KP pathway cause oxidative damage to the brain (Bonda et al. 2011). Increased QUIN led to a concentration-dependent increase in ROS in the synaptosomes of rat brains and

lipid peroxidation in the hippocampus (Santamaría et al. 2001). Researchers have also shown that QUIN can increase nitric oxide synthase activity more than three-fold (Pérez-Severiano et al. 1998). This enzyme produces nitric oxide, a vaso-relaxing free radical. Amyloid-beta production increased the concentration of QUIN (Guillemin et al. 2003), linking AD more directly to oxidative damage from amino acid metabolites. Moreover, a shift in tryptophan degradation to the KP pathway diverts tryptophan from the serotonin synthesis pathway. This could deprive the AD brain of serotonin, contributing to the pathogenesis of AD. However, knowledge of aromatic amino acid metabolism and signaling in AD brain is far from complete. Dietary tryptophan restriction has been shown to extend the lifespan of rodents (Segall 1977), but the mechanism has not been investigated. Acute tryptophan depletion leads to memory impairment (Riedel et al. 2002).

The amino acid tyrosine is important for synthesizing catecholamines, but there are only a few studies measuring tyrosine levels specifically in AD brain, although several studies have found that oral tyrosine administration improves memory and cognitive function (van de Rest et al. 2013). Phenylalanine can be metabolized through the same metabolic pathways as tyrosine but only when tyrosine levels are low (Fernstrom and Fernstrom 2007), preventing firm conclusions regarding changes in phenylalanine levels in AD brain until changes in tyrosine levels are measured as well. As a class, aromatic amino acid metabolism is especially important for neural functioning, and more research is needed to elucidate the relevance of the changes that occur in AD.

Charged Amino Acids

The charged amino acids include the acidic (aspartate and glutamate) and basic (arginine, lysine, and histidine) amino acids. Each of these appears to be decreased in AD patient brain or plasma, with the possible exception of glutamate where the direction of change may depend upon the brain region assayed. Glutamate (G. Wang et al. 2014), histidine, and aspartate levels were decreased in serum from AD patients (Gonzalez-Dominguez et al. 2015), while aspartate and glutamate levels were decreased in the temporal lobe of the cerebral cortex of AD patients (Gueli and Taibi 2013). Xu *et al*. measured a decrease in both lysine and aspartate levels in the brains from autopsied AD patients, while glutamate levels increased (Xu et al. 2016). Glutamate's interaction with the NMDA receptor is critical for learning and memory formation (Esposito et al. 2013), but glutamate excitotoxicity also leads to neuronal death in AD (Mattson et al. 1992; Hynd et al. 2004). As discussed below, aspartate and glutamate also play a role in transamination reactions such as those occurring upstream of the urea cycle. As a group, the levels of charged amino acids are altered in AD, suggesting specific perturbations in metabolism, but more research needs to be done to determine the cause of the changes in amino acid levels and the effects these have on the brain. Examining the activity of more enzymes involved in amino acid metabolism and building computational models of amino acid metabolism could help explain the alterations in amino acid levels in AD.

Glutamine

Glutamine is the most prevalent amino acid in plasma and, together with glutamate, the most prevalent amino acids in human brain (Cooper and Jeitner 2016). Glutamate

supplementation decreases tau phosphorylation and has shown other protective effects in a mouse model of AD (Chen and Herrup 2012). It has been found that glutamine, glutamate, aspartate, alanine, and purines are likely degraded as the top alternative energy sources in neurodegenerative diseases such as AD when glucose metabolism is disturbed (Kori et al. 2016). These amino acids are readily broken down because high levels of aminotransferases for the initial step in the catabolism of alanine, aspartate, and glutamate are present in brain (Cooper and Jeitner 2016). In AD patients, glutamine levels have been shown to decline in the serum (González-Domínguez et al. 2015) but increase in the temporal cortex of the brain (Gueli and Taibi 2013). Glutamine and alanine levels have also been found to be decreased in the blood in transient global amnesia (Sancesario et al. 2013).

There has not been much data generated on how the brain maintains balances in amino acid and total nitrogen levels during times of neuronal amino acid catabolism, but it is likely that BCAAs can be taken up through the blood-brain barrier (BBB) and glutamate can be released to maintain nitrogen balance (Hawkins and Viña 2016). BCAA-derived carbons can then be fed into the citric acid cycle to form alpha-ketoglutarate, and then the alpha-ketoglutarate can be transaminated to glutamate to maintain glutamate levels. During times when ammonia levels increase in the brain, exporting a glutamine (containing two nitrogen atoms) from the brain for every BCAA (or other amino acid containing a single nitrogen atom) taken up would allow for a net efflux of nitrogen to lower the brain ammonia levels (Cooper and Jeitner 2016).

Sulfur-Containing Amino Acids

The sulfur containing amino acids are cysteine and methionine. Much research has been performed on supplementation with cysteine and the more membrane permeable form N-acetylcysteine (NAC) as cysteine levels limit the synthesis of glutathione, one of the most important antioxidants in the body. NAC appears to cross the blood-brain barrier slowly, so other therapies are under development to increase brain glutathione levels (Bavarsad-Shahripour et al. 2014). There have been three small clinical trials of NAC supplementation to AD patients with mixed results (Deepmala et al. 2015). Therefore, more research is needed to clarify the effects of NAC on AD. There seems to be a disruption in sulfur-containing amino acid metabolism in AD as serum and brain homocysteine levels increase (Hu et al. 2016). Cysteine levels were also shown to be increased in the hippocampus of autopsied AD patients (Xu et al. 2016). Homocysteine-cysteine disulfide levels were also found to increase in AD patient serum while methionine levels decreased (González-Domínguez et al. 2014). Increased homocysteine levels in the plasma is a known risk factor for AD and other dementias (Seshadri et al. 2002). Dietary methionine supplementation caused increased amyloid-beta and phosphorylated tau levels in brain and cognitive impairment in wild-type mice (Tapia-Rojas et al. 2015). Dietary methionine restriction led to decreased amyloid-beta levels and neuroprotection in APP-PS1 AD mice (Sambamurti et al. 2015) and decreased mitochondrial complex I-mediated superoxide production and increased lifespan in rats (Orentreich et al. 1993) while cysteine supplementation led to a slight decrease in mTOR activity (Gomez et al. 2015). However, cysteine supplementation prevented the decreased ROS production in methionine restricted animals.

Amino Acids as an Energy Source in AD Neurons

One of the hallmarks of AD is dysfunctional energy metabolism. Mitochondrial-derived oxygen free radicals produced in AD brain are known to damage glycolytic enzymes such as enolase (Butterfield and Lange 2009) and glyceraldehyde-3-phosphate dehydrogenase (Butterfield et al. 2010), slowing glycolysis. This damage, combined with decreased insulin signaling in AD brains (Calvo-Ochoa and Arias 2015), results in decreased glucose uptake and metabolism which has been confirmed as an early event in AD progression through the use of fluorodeoxyglucose (FDG)-PET scans (Mosconi et al. 2010). The decreased glucose metabolism results in decreased pyruvate production which, combined with amyloid-beta-mediated mitochondrial complex IV inhibition (Wong-Riley et al. 1997) and tau-mediated mitochondrial complex I inhibition (Rhein et al. 2009), results in decreased mitochondrial energy metabolism and ATP levels. Most cells in the body show metabolic flexibility and can increase mitochondrial fatty acid beta-oxidation when glycolytic output decreases to maintain cellular ATP levels. Neurons contain very low levels of fatty acid beta-oxidation enzymes (Panov et al. 2014), so they instead rely upon ketone body catabolism, amino acid catabolism, or catabolism of lactate released from astrocytes (Mächler et al. 2016) to maintain cellular ATP levels. Ketone body levels are normally very low in the well-fed and unexercised human body (Sleiman et al. 2016). Therefore, amino acid catabolism together with lactate metabolism and limited glucose metabolism likely play essential roles in maintaining cellular ATP levels in AD neurons. Data supporting this hypothesis come from clinical studies that show a 44% decrease in glucose utilization in autopsied early-onset familial Alzheimer's disease brain. The AD brains showed no deficits in oxygen utilization as free amino acids (and perhaps lactate)

were oxidized for energy generation in replacement of glucose, leading to decreased amino acid levels (Hoyer et al. 1988). Once brain amino acid levels were depleted, brain ammonia levels decreased as well. These data indicate that amino acid supplementation or high protein diets may help to energize the AD brain.

The most abundant amino acids present in the human brain as a potential energy source are glutamate and glutamine, present at roughly 7-8 mM, while the next most abundant amino acids are aspartate and taurine which are present at roughly 1.2 mM, and then serine, GABA, and glycine that are present at roughly 0.5 mM (Cooper and Jeitner 2016). The brain contains a glutamate-glutamine cycle where glutamate is released by neurons into synaptic clefts; the glutamate is then taken up by astrocytes where some is broken down for energy but most is converted to glutamine which is exported from the astrocytes and taken up once again by neurons and converted back into glutamate. To facilitate this cycle, the brain has been shown to possess high levels of two neuronal glutaminase genes, GLS and GLS2 (phosphate-activated mitochondrial glutaminase), to function in the breakdown of glutamine to glutamate that releases ammonia, but small amounts of glutaminase (mostly GLS) have also been localized to astrocytes (Cardona et al. 2015). GLS2 activity is strongly upregulated by ADP (Masola and Ngubane 2010) and has been shown to decline with aging (Walton and Dodd 2007), particularly in AD brain (Akiyama et al. 1989).

Further release of ammonia in the brain can occur if glutamate is catabolized to alpha-ketoglutarate by glutamate dehydrogenase. In addition to the normal mammalian glutamate dehydrogenase gene, GLUD1, primates have a second glutamate dehydrogenase gene, GLUD2, that is expressed in astrocytes and may be needed in those cells to preserve

alpha-ketoglutarate levels in the presence of high glutamine synthetase activity (Spanaki and Plaitakis 2012). Glutamine synthetase activity has been shown to decline in AD brain (Smith et al. 1991). Mitochondrial GLUD1 is negatively regulated by the GTP formed from citric acid cycle function, while GLUD2 is relatively unaffected by guanine nucleotides but is positively regulated by ADP and branched chain amino acids (Zaganas et al. 2014). The negative regulation of GLUD1 when energy levels are high allows for preservation of glutamate levels needed for neurotransmission as well as prevents the toxic buildup of ammonia. Glutamate can also be metabolized to alpha-ketoglutarate to fuel citric acid cycle metabolism without the release of free ammonia through the function of the alanine, aspartate, and branched chain aminotransferases (McKenna et al. 2016) if the corresponding ketoacids are present in adequate amounts.

Normally both neurons and astrocytes oxidize glucose by glycolysis and the resulting pyruvate by oxidative phosphorylation to maximize ATP yield (Patel et al. 2014). However, there is much evidence that indicates astrocytes are more metabolically flexible than neurons due to their slightly lower energy demands (Stobart and Anderson 2013). Therefore, astrocytes can survive predominately by glycolysis with little oxidative metabolism. In metabolically stressful times, such as in AD, astrocytes may convert the pyruvate produced from glycolysis into lactate to maintain the cellular redox state and then export the lactate which can then be taken up by neurons, converted back to pyruvate, and used for oxidative energy metabolism, a process called the astrocyte-neuron lactate shuttle (Pellerin and Magistretti 2012). Astrocytes may also be able to increase the amount of glutamine released to neurons to be utilized as an energy source under these conditions when neuronal glycolysis is impaired. Astrocytes possess some fatty-acid beta-oxidation

capabilities (Panov et al. 2014) which likely becomes important during these times when neuronal energy metabolism is impaired.

A study using AD mice found that a high protein/low carbohydrate diet resulted in a 5% reduction in the brain weight in AD mice, including decreased neuronal density and volume in the CA3 region of the hippocampus that is important for memory (Pedrini et al. 2009). A high protein/low carbohydrate diet has also been associated with increased excitotoxicity in the aged brain (Pal and Poddar 2008). These data suggest that high levels of amino acids or products of their catabolism may contribute to neurodegeneration. Consistent with this assessment, catabolism of branched chain keto acids by branched chain ketoacid dehydrogenase (BCKDH) in mitochondria results in substantial production of damaging superoxide radical (Quinlan et al. 2014). In addition, mice on a low protein/high carbohydrate diet lived longer than those on a high protein/low carbohydrate diet and had lower insulin levels and lower mTOR activation (Solon-Biet et al. 2014). However, the health benefits of a low protein/high carbohydrate diet may only extend through middle age as aged mice and humans on a high protein diet showed protection from disease (Levine et al. 2014). Therefore, amino acid supplementation therapies may best be explored as therapies for late-onset AD. Interestingly, plasma levels of all three branched chain amino acids showed a positive correlation with dietary protein intake in mice, while the plasma levels of most other amino acids showed a negative correlation with dietary protein intake. Therefore, some of the beneficial health effects conferred by the low protein diet in young and middle aged mice may be mediated by decreased plasma branched chain amino acid levels.

The Urea Cycle and AD

Amino Acid Metabolism, Ammonia, and the Urea Cycle

Proteins are digested in the stomach and intestine by a variety of peptidases into free amino acids and dipeptides; the dipeptides are further catabolized into amino acids by first pass hepatic metabolism. These amino acids are then transported by the blood to other tissues where the amino acids are used for protein synthesis or broken down in processes that produce ammonia when levels exceed their requirements. The α-amino group of the amino acid is often transferred to α-ketoglutarate to form glutamate and an α-keto acid. Glutamate can undergo oxidative deamination to form ammonia and α-ketoglutarate, or the amino group can be transferred to oxaloacetate to form aspartate and α-ketoglutarate. Aspartate is required for urea cycle function. In the brain and muscle (tissues normally lacking appreciable urea cycle function) aspartate can enter the purine nucleotide cycle to release fumarate and ammonia. Other reactions produce ammonia as well; histidine, serine, threonine, and tyrosine-derived catecholamine catabolism each release ammonia through separate reactions. Ammonia is toxic and needs to be eliminated quickly or converted to a less toxic form. Once ammonia and glutamate combine to form glutamine through the action of the glutamine synthetase enzyme, the glutamine is exported from the tissue and transported through the blood to the liver where the free ammonia is released through the action of the glutaminase enzyme. The urea cycle can then function to convert the ammonia to urea, which is excreted from the body.

The first and second steps of the urea cycle occur in the mitochondria, while the other three steps occur in the cytoplasm. First, ammonia combines with CO_2, H_2O, and HCO_3^- to form carbamoyl phosphate. N-acetylglutamate is required as a cofactor for this

reaction to proceed. Carbamoyl phosphate reacts with ornithine to produce citrulline, which is transported out of mitochondria and then reacts with aspartate to form argininosuccinate. Arginosuccinate is converted by argininosuccinase into fumarate and arginine. In the final step, arginase converts arginine into ornithine and urea. **Figure 5.1** summarizes amino acid catabolism leading up to and including the urea cycle.

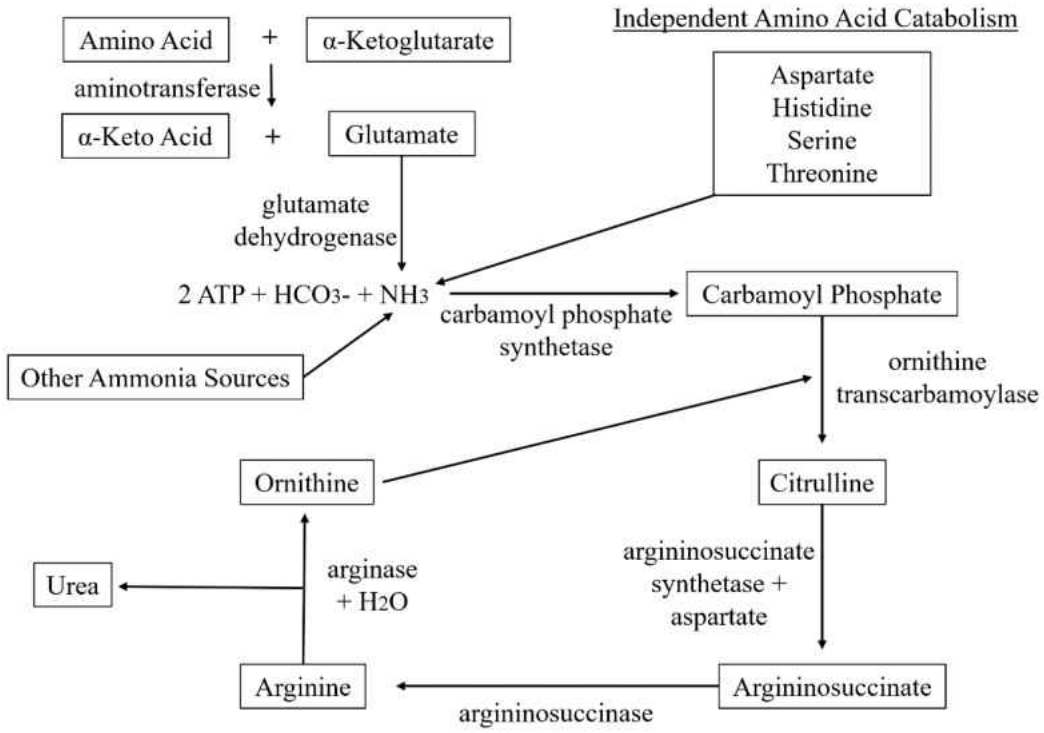

Figure 5.1: Overview of the urea cycle. Ammonia produced as a result of metabolic processes involving amino acids and other biomolecules is converted into nontoxic urea in the urea cycle for excretion. The intermediates are in boxes and the enzymes and other necessary substrates are next to the arrows in lowercase. These intermediate metabolite and enzyme levels are altered in the brain in Alzheimer's disease.

Changes in Components of the Urea Cycle in AD

Levels of enzymes and metabolic intermediates of the urea cycle are altered in patients with AD. It is widely accepted that the urea cycle occurs almost exclusively in the liver and intestines (Takiguchi and Masataka 1995). It has been shown that normal human brain has low or no ornithine transcarbamoylase (OTC) activity, thus preventing urea cycle activity (Bensemain et al. 2009). Carbamoyl phosphate synthetase activity is also low in brain tissue. However, studies using autopsied brains from AD patients have challenged this exclusive localization of the urea cycle. Hansmannel and colleagues identified mRNA expression for all enzymes of the urea cycle in the brains of both normal adults and patients with AD (Hansmannel et al. 2010). However, the mRNA levels of OTC were extremely low in the non-AD subjects, and the normal cytoplasmic urea cycle enzyme arginase I was extremely low in both populations. Arginase is one of the more studied urea cycle enzymes that appears to be dysregulated in AD. Arginase converts arginine to urea and ornithine (see Figure 5.1). Two groups, Lui et al. (P. Liu et al. 2014) and Hansmannel et al. (Hansmannel et al. 2010), found the same trend of increased mitochondrial arginase II (Arg2) levels in autopsied AD patient brain. Hansmannel et al. used RT-PCR to find a 55% increase in Arg2 mRNA levels in AD patients compared to controls (Hansmannel et al. 2010), whereas Lui et al. used Western blot to show an increase in the total amount of Arg2 protein in two different brain regions with no change in a third (P. Liu et al. 2014).

There are several important consequences of increased Arg2 expression in AD brain. First, increased arginase activity increases urea and ornithine levels, the latter being a precursor of polyamine synthesis. Polyamines can play an important neuroprotective

role in the brain. Second, increased arginase activity decreases arginine levels, which can lead to decreased mTOR activity. Arginine is also a substrate for nitric oxide synthase which produces the vasorelaxing free radical nitric oxide that can increase neuroinflammation. Therefore, transgenic overexpression of arginase I showed neuroprotection in a tau-overexpressing model of AD (Hunt et al. 2015). However, an arginase inhibitor showed neuroprotective effects in an amyloid-beta-producing mouse model of AD (Kan et al. 2015). Therefore, it is possible that arginase expression has different effects on amyloid and tau pathology. Arg2 is the main isoform in AD brain and is highly expressed in endothelial cells. Therefore, it is also possible that arginase I activity is neuroprotective while arginase II activity is neurotoxic due to expression in different cell types or different subcellular localizations.

Bensemain et al. used RT-PCR to detect the transcription of the ornithine transcarbamylase (OTC) gene and other enzymes of the urea cycle in AD brains as well (Bensemain et al. 2009). OTC activity was exclusively localized to brain endothelial cells, and its activity in cerebrospinal fluid was nearly 9 times higher in AD patients than in the control group (Bensemain et al. 2009). It is interesting that OTC activity was concentrated in the endothelia in the vasculature of the brain in AD (Bensemain et al. 2009); these areas are severely affected by amyloid plaques (Koizumi et al. 2016). Taken together, these results indicate that the urea cycle may occur in the endothelial cells of AD patients, but this may rely upon the transport of arginine from the cytoplasm to the mitochondria to be metabolized by arginase II. The mitochondrial ornithine carriers ORC1, ORC2, and SLC25A29 are also able to transport arginine (Monné et al. 2015). ORC1 and ORC2 are

expressed at very low levels in brain (Begum et al. 2002), but this may be enough to allow low level urea cycle activity in the endothelial cells from AD patients.

Perhaps the most notable urea cycle metabolite change in the AD brain is in the level of urea. The direction of the change in level of urea depends on the clinical or pathological sample or the mouse model tested. Serum from human AD patients showed a 44% decrease in urea levels when assayed using GC/MS (Gonzalez-Dominguez et al. 2015). The same group found decreased urea in the serum of APP/PS1 AD model mice (González-Domínguez et al. 2015). A decrease in urea in the hippocampus of the senescence-accelerated SAMP8 mice was also measured (H. Wang et al. 2014). SAMP8 mice show neurodegeneration similar to that observed in AD. The decreased urea levels are consistent with decreased arginase levels found in APP/PS1 mouse brain (Tapia-Rojas et al. 2015). Data collected from studies of human brain tell a completely different story. A study by Gueli and Taibi using GC/MS on temporal lobe extracts demonstrated that urea was increased in brain tissue of AD patients over 2-fold (Gueli and Taibi 2013). Xu and colleagues measured urea in six different regions of the brain to find that urea was increased in AD patients' brains by an average of more than 5-fold (Xu et al. 2016). This increase in urea levels is consistent with the increased Arg2 levels in human AD brain. Interestingly, in the striatum of post-mortem Huntington's disease brain, urea was found to be the most decreased (3.2-fold) metabolite (Graham et al. 2016), but another study found opposite results that urea was increased in all brain regions examined in post-mortem Huntington's patients (Patassini et al. 2015).

Ornithine levels were decreased in AD brain and serum (P. Liu et al. 2014; Gonzalez-Dominguez et al. 2015; Xu et al. 2016). Although ornithine is the product of an enzyme that

is upregulated (Arg2), the decrease is consistent with other findings because ornithine is the substrate of OTC, another upregulated enzyme in AD brain (Bensemain et al. 2009), and ornithine is a precursor for the production of polyamines that increase in abundance in AD brain (Guerra et al. 2016). Citrulline, however, appears to remain unchanged in AD brains (P. Liu et al. 2014; Pan et al. 2015). Citrulline is a strong antioxidant and citrulline supplementation prevented age-related changes in lipid metabolism in mouse hippocampus (Marquet-de Rougé et al. 2013). Aspartate reacts with citrulline to form argininosuccinate. Aspartate levels are decreased in AD patient serum (Gonzalez-Dominguez et al. 2015), and both aspartate and arginine levels are decreased in the brain of AD patients (Gueli and Taibi 2013; Xu et al. 2016). Decreased levels of urea cycle intermediates could indicate their efficient metabolism. Considering that different groups have shown increased urea levels in biopsied AD brains as well as increased expression of one or more urea cycle genes, current evidence suggests that urea cycle activity may be induced in endothelial cells from AD patient brain. It is possible that a urea cycle metabolite such as arginine that is produced in neurons and glia is imported into AD endothelial cells where Arg2 levels are high and OTC is exclusively present to finish the urea cycle there. The citrulline produced from endothelial cell OTC activity could also be exported to neurons or glia to finish the urea cycle. However, it is also possible that the higher urea levels found in AD brain are strictly due to increased Arg2 levels independent of complete urea cycle function.

Increased urea levels in AD brain raises questions as to what could be leading to the increased expression of OTC and arginase II. The main function of the urea cycle is to safely dispose of nitrogenous waste from amino acid catabolism and other sources. Therefore, it

has been hypothesized that abnormal nitrogen metabolism may play a role in the pathogenesis of AD (Seiler 1993). One of the early hypotheses for the pathogenesis of AD, proposed by Seiler in 1993, was the ammonia hypothesis; this posits that increased levels of ammonia accumulate in and are toxic to the AD brain (Seiler 1993). However, the amyloid cascade hypothesis was proposed the year before (Hardy and Higgins 1992), and the ammonia hypothesis was not thoroughly investigated (Seiler 2002). The ammonia hypothesis of AD was generated due to the following observations: increased ammonia levels measured in the plasma from AD patients (Fisman et al. 1985; Hoyer et al. 1990), decreased glutamine synthetase enzyme levels in AD astrocytes to scavenge ammonia (Smith et al. 1991; Le Prince et al. 1995), increased adenosine deaminase activity in AD brain (Sims et al. 1998), and increased monoamine oxidase activity in AD brain (Nakamura et al. 1990; Jossan et al. 1991); the latter two enzymes produce ammonia. Ammonia has also been implicated as a cause of oxidative damage in the brain as it was found to increase reactive oxygen species levels in SH-SY5Y cells (Bobermin et al. 2015) and astrocytes (Murthy et al. 2001) and lead to RNA oxidation in rats (Görg et al. 2008). Furthermore, mitochondrial activity in rat and mouse models appears to be impaired by ammonia. Ammonium acetate injection into rats led to decreased state III respiration (Kosenko et al. 1997), cytochrome c oxidase activity (Rama-Rao et al. 1997), and decreases in various other respiratory complexes in isolated synaptic mitochondria (Qureshi et al. 1998). Impaired mitochondrial function is often associated with increased oxidative damage. This may in part explain the increase in reactive oxygen species in the presence of ammonia. Increased ammonia production would either necessitate urea cycle function to metabolize the toxic ammonia to urea or necessitate increased reaction of ammonia with glutamate

catalyzed by astrocytic glutamine synthetase followed by export of glutamine from the brain. Evidence from studies of the urea cycle and amino acid metabolism in AD subjects and mouse models justifies further investigation into the production and detoxification of ammonia in the AD brain.

Considerations for Dietary Metabolite Supplementation as a Treatment for AD

Increasing or decreasing the levels of specific amino acids and other metabolites in the diet has shown some promise in improving markers of aging and longevity (Fernstrom 2005), so it is possible that nutrient supplementation or restriction may improve neural functioning in AD patients as age is the major risk factor for AD. However, there are several hurdles to overcome before an efficacious treatment can be formulated. For example, studies on intestinal transport, bioavailability, hepatic metabolism and excretion, and blood-brain barrier transport are needed in order to choose the optimal formulations. Much of this information is present for a few commonly studied amino acids, but much of it is absent for the vast majority of amino acids. From what is known, it appears that hepatic metabolism, especially of amino acids, presents the greatest challenge to overcome for the supplementation of many of the amino acids for their use in the treatment of neurodegeneration, but intestinal transport may also become limiting in the elderly (Levine et al. 2014). Several of the amino acids also have limited blood-brain permeability. We will present one promising strategy taking these many challenges into consideration.

As mentioned above, intestinal uptake of amino acids declines past age 65. It has been shown that the bioavailability of individual amino acids and dipeptides is slightly better than that of amino acids consumed as polypeptides since the individual monomers

can be absorbed quickly without the need for further enzymatic hydrolysis in the gut. Therefore, dietary supplementation with individual amino acids or combinations of individual amino acids would likely benefit the elderly in addition to a high protein diet that promotes health in the elderly (Levine et al. 2014). The use of individual amino acids also has the added benefit of being able to stimulate specific signaling pathways. Due to the limited intestinal uptake of amino acids in the elderly, they may particularly benefit from supplementation with hydrophobic, more membrane permeable forms of amino acids such as amino acid ethyl esters or N-acetyl amino acids. These amino acid derivatives show a greater probability of diffusion across membrane bilayers such as intestinal epithelia and the capillary endothelia of the blood-brain barrier where the activity of specific membrane transporters may be limiting. These more hydrophobic amino acid derivatives are cleaved by esterases and other hydrolytic enzymes intracellularly or extracellularly to release the free amino acid. This hydrolysis may occur to a large extent during first pass hepatic metabolism.

Dietary aspartate, glutamate, and glutamine are oxidized as primary sources of fuel for intestinal cells (Reeds et al. 2000). In addition, these amino acids, especially glutamate and aspartate, are transported very poorly through the blood-brain barrier (Hawkins et al. 2006) even though they are present at high concentrations in the brain. Other amino acids that are transported poorly through the blood-brain barrier include glycine, alanine, proline, and GABA. Medium and large side chain, non-polar amino acids are transported readily by the blood-brain barrier into the brain including aromatic amino acids, BCAAs, methionine, histidine, and threonine (Smith 2000). These amino acids all compete for transport through the L1 transport pathway. Glutamine and asparagine are also likely

transported by this pathway. Another amino acid transporter called the y^+ system transports basic amino acids such as arginine, lysine, and ornithine as well as many neutral amino acids such as serine through the blood brain barrier (Hawkins et al. 2006). One potential therapeutic strategy for AD patients is to supplement their diets with high levels of BCAAs, aromatic amino acids, and threonine. Through competition for the L1 amino acid transport system, this therapy could limit the transport of methionine into the brain, perhaps yielding the known metabolic and neuroprotective benefits of methionine restriction (Sanchez-Roman and Barja 2013; Tapia-Rojas et al. 2015). However, methionine is also transported into the brain to a limited extent through the y^+ system; this may hinder the effectiveness of this strategy. A second potential therapy is to omit leucine and/or isoleucine from the previously mentioned supplementation strategy. These two amino acids are not transported by the y^+ system (Hawkins et al. 2006). Depletion of leucine or isoleucine in the brain would lead to amino acid imbalance, activation of GCN2 kinase, and possibly the inhibition of mTOR kinase to slow protein translation rates that may be beneficial for reducing the levels of neurofibrillary tangles formed from hyperphosphorylated aggregates of tau protein. These amino acid supplementation therapies could be combined with supplementation with other metabolic fuels such as D-beta-hydroxybutyrate (a ketone body), citric acid cycle intermediates, pyruvate, and/or lactate, which would decrease the reliance of AD neurons on the use of amino acids as a fuel. Consumption of high levels of these alternative metabolic fuels may function to restore neuronal amino acid levels.

Summary and Conclusions

Dietary amino acids provide a large amount of carbon and nitrogen to the body that can be metabolized by a myriad of biochemical pathways. Amino acids have roles in neuronal signaling, energy production, and nitrogenous waste production and elimination. These processes are important for normal physiology, so it is not surprising that disease states result from major metabolic dysfunction, but whether relatively minor perturbations of this metabolism contribute to neurodegeneration requires further study. Brains and serum from AD patients have consistently shown alterations in amino acid levels and metabolism that could provide a basis for some of the symptoms of the disease. These individual changes may each play a different role in the disease, highlighting the complexity that underlies AD pathology. An increase in urea in the brains of AD patients together with the altered expression of urea cycle enzymes suggests that urea cycle activity is induced in AD brain endothelial cells. Viewing AD as a metabolic disease provides valuable insight into possible new targets for drug discovery in the AD research field. A summary of some of the altered amino acid metabolism that occurs in AD is shown in **Figure 5.2.**

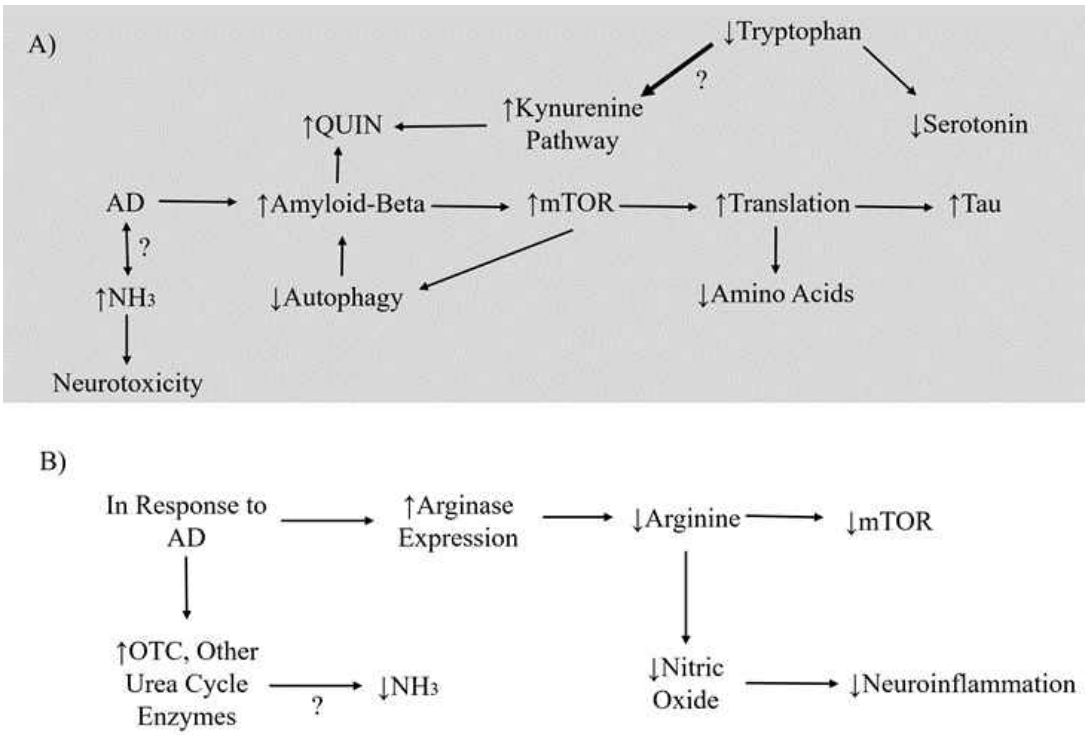

Figure 5.2: Pathogenic processes and proposed physiological responses in Alzheimer's disease. A) An overview of the pathogenic processes of Alzheimer's disease. B) An overview of how the body may respond to AD to minimize damage by changing the expression of enzymes involved in amino acid catabolism.

The measurement of metabolite levels provides a snapshot of a very dynamic process. While this information is extremely useful, it is not sufficient by itself to understand the pathological changes associated with AD. Further studies measuring enzyme activities could provide complementary information about the dynamics of amino acid metabolism in AD. In addition, studies overexpressing OTC and Arg2 to activate the urea cycle in the brain endothelial cells of an AD mouse model would help clarify the effects of endothelial urea cycle activity on brain physiology and cognitive function. Studying AD from a metabolic perspective could lead to dietary supplementation therapies that could delay disease progression or alleviate some of the suffering caused by the disease.

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CHAPTER 6

IN SILICO PRELIMINARY ASSOCIATION OF AMMONIA METABOLISM GENES *GLS*, *CPS1*, AND *GLUL* WITH RISK OF ALZHEIMER'S DISEASE, MAJOR DEPRESSIVE DISORDER, AND TYPE 2 DIABETES

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Abstract

Ammonia is a toxic byproduct of protein catabolism and is involved in changes in glutamate metabolism. Therefore, ammonia metabolism genes may link a range of diseases involving glutamate signaling such as Alzheimer's disease (AD), major depressive disorder (MDD), and type 2 diabetes (T2D). We analyzed data from a National Institute on Aging study with a family-based design to determine if 45 single nucleotide polymorphisms (SNPs) in glutaminase (*GLS*), carbamoyl phosphate synthetase 1 (*CPS1*), or glutamate-ammonia ligase (*GLUL*) genes were associated with AD, MDD, or T2D using PLINK software. HAPLOVIEW software was used to calculate linkage disequilibrium measures for the SNPs. Next, we analyzed the associated variations for potential effects on transcriptional control sites to identify possible functional effects of the SNPs. Of the SNPs that passed the quality control tests, four SNPs in the *GLS* gene were significantly associated with AD, two SNPs in the *GLS* gene were associated with T2D, and one SNP in the *GLUL* gene and three SNPs in the *CPS1* gene were associated with MDD before Bonferroni correction. The *in silico* bioinformatic analysis suggested probable functional roles for six associated SNPs. Glutamate signaling pathways have been implicated in all these diseases, and other studies have detected similar brain pathologies such as cortical thinning in AD, MDD, and T2D. Taken together, these data potentially link *GLS* with AD, *GLS* with T2D, and *CPS1* and *GLUL* with MDD and stimulate the generation of testable hypotheses that may help explain the molecular basis of pathologies shared by these disorders.

Keywords

Ammonia, Glutamate, Alzheimer's Disease, Major Depressive Disorder, Type 2 Diabetes

Background

Excess dietary protein is catabolized to release ammonia. Because of the relative toxicity of ammonia (Auron and Brophy 2012), it is removed from the body by the urea cycle and excreted as the relatively non-toxic compound urea. The urea cycle is thought to occur almost exclusively in the liver (Dimski 1994). However, certain types of ammonia metabolism such as glutamate cycling can occur in other tissues. Among the enzymes involved in ammonia metabolism are glutaminase (GLS, EC 3.5.1.2), carbamoyl phosphate synthetase 1 (CPS1, EC 6.3.4.16), and glutamate-ammonia ligase (GLUL, EC 6.3.1.2), also known as glutamine synthetase. CPS1 is the first committed step of the urea cycle. Individuals who lack a functional *CPS1* gene have severe hyperammonemia resulting in cognitive impairment (Klaus et al. 2009). The majority of cells in the body (such as those in the brain) that lack a functional urea cycle rely on GLUL to locally remove ammonia by ligating it to glutamate to form glutamine (Cooper and Jeitner 2016). Glutaminase catalyzes the reverse process, releasing ammonia from glutamine to form glutamate. The human genome contains two glutaminase genes. Data from the Human Protein Atlas (www.proteinatlas.org) suggest *GLS* is expressed primarily in the brain and kidney (http://www.proteinatlas.org/ENSG00000115419-GLS/tissue) (Uhlén et al. 2015). Because glutamate is an important neurotransmitter and signaling molecule, both GLUL and GLS are necessary for proper neural functioning (Cooper and Jeitner 2016).

Because (1) glutamine and glutamate are abundant and ubiquitous amino acids involved in ammonia metabolism, (2) ammonia is a byproduct of protein catabolism, and (3) glutamate is an important signaling molecule, we hypothesized that the *CPS1*, *GLUL*, and *GLS* ammonia metabolism genes could influence various disease processes across a range of tissues. Evidence for a link between ammonia metabolism genes and type 2 diabetes (T2D) includes studies that have found changes in *GLS* activity in a rat model of diabetes (Ardawi 1987). In addition, a SNP in the *GLUL* gene has been associated with all-cause mortality in individuals with T2D (Prudente et al. 2015), and a separate study has found a transcriptomic link between T2D and *GLUL* (Mirza et al. 2014). Ammonia and glutamate metabolism changes have also been linked to mood disorders such as major depression. For example, glutamate signaling pathways have been shown to be altered in major depressive disorder (MDD) (Bernard et al. 2011). Furthermore, *GLUL* expression has been shown to be changed in individuals with MDD (Choudary et al. 2005; Miguel-Hidalgo et al. 2010; Bernard et al. 2011). However, one study reports no change in GLUL activity in astrocytes from the brains of individuals with MDD (Chandley et al. 2013). Further evidence supporting this connection is that researchers were able to induce behaviors consistent with depression in mice by inhibiting enzymes involved in ammonia and glutamate metabolism (Lee et al. 2013).

The potential link between ammonia metabolism and Alzheimer's disease (AD) is particularly well-supported. The amyloid cascade hypothesis is the dominant hypothesis for the pathogenesis of AD (Hardy and Higgins 1992). However, increasing evidence suggests increased amyloid-beta levels are only one facet of this complex disease (Herrup 2015). The ammonia hypothesis for the etiology of AD was first proposed in 1993 (Seiler

1993), but it has not yet been thoroughly investigated. The ammonia hypothesis suggests ammonia toxicity plays a causal role in AD. This is supported by several observations reviewed by Seiler (Seiler 2002). Briefly, individuals with AD have been found to have increased blood ammonia (Fisman et al. 1985) and cerebrospinal fluid ammonia levels. The negative effects of ammonia on cognitive functioning are well documented (Raabe 1987), and studies have suggested that there are high ammonia concentrations in AD brain (Hoyer et al. 1990). AD patients have also been shown to have increased plasma glutamate compared to controls (Miulli et al. 1993). Further studies suggest changes in the activity of ammonia metabolism genes may be involved in AD. Researchers have found changes in *GLUL* expression in AD patients (Robinson 2000) as well as in mouse models of AD (Kulijewicz-Nawrot et al. 2013). Changes in *GLS* expression in AD brains have been found as well (Akiyama et al. 1989; McGeer et al. 1989; Burbaeva et al. 2014). The aforementioned studies suggest an association between *GLUL*, *GLS*, and *CPS1* genes and disorders such as AD, T2D, and MDD. While all three of the genes of interest have not been explicitly implicated in all three diseases, there is sufficient evidence for the involvement of ammonia metabolism genes in the pathologies of these diseases to warrant further investigation.

All three of these diseases have genetic heritability; AD is estimated to have a heritability of 0.74 (Gatz et al. 1997), MDD of 0.37 (Sullivan et al. 2000), and T2D of 0.47-0.77 (Willemsen et al. 2015). There is also ample epidemiological evidence of associations among MDD, T2D, and AD. T2D has been found in several studies to be linked to the incidence of AD (Ott et al. 1999; Luchsinger et al. 2005; Gudala et al. 2013; Li et al. 2015). Two separate analyses of a GWAS found SNPs that were associated with both T2D and AD

(Hao et al. 2015; Gao et al. 2016), suggesting the possibility of a shared genetic etiology. However, none of the hits from these studies were the genes we tested. There is also epidemiological evidence of an association between AD and MDD. Individuals with a history of depression were shown to have an increased risk for AD (Geerlings et al. 2008). Finally, T2D has been shown by several studies to nearly double the risk of developing MDD (Anderson et al. 2001; Ali et al. 2006). However, not every study supports a genetic association amongst these diseases. A study of 32 genetic variants identified in GWAS studies of individuals with T2D found no association with AD (Chung et al. 2015). Another study also found no association between SNPs associated with T2D and risk of AD (Proitsi et al. 2014). A third study found no common genetic variants between individuals with AD and MDD (Gibson et al. 2017). Studies that continue to investigate the genetic component of these diseases could help untangle knowledge of genetic associations and elucidate targets for novel treatment strategies.

To investigate the potential association between these select ammonia metabolism genes and AD, MDD, and T2D, we used data from a family-based study of Alzheimer's disease patients. The variations significantly associated with one or more of these diseases were interpreted considering previously published experimental results and bioinformatics analysis of possible effects of variants on gene expression. This study generates several hypotheses useful for guiding future work into mechanisms of pathogenesis of AD, MDD, and T2D and investigations into how these mechanisms may interact and overlap.

Methods

NIA-LOAD Family Study Subjects

Data for this study came from the National Institute on Aging—Late Onset Alzheimer's Disease Family Study: Genome-Wide Association Study for Susceptibility Loci—Study Accession: phs000168.v1.p. There were 3,007 individuals selected consisting of 1266 AD, 247 T2D, and 1688 MDD individuals, and 1279 non-AD individuals from 1386 pedigrees (589 nuclear families). Details on these subjects have been previously published (Lee et al. 2008). Population stratification does not apply to a family-based study design. The number of individuals in this data set with each type of disease and combinations of co-occurrences are shown in **Table 6.1**.

Table 6.1 Number of individuals with co-occurrence of AD, MDD, and T2D in the data set[a]

AD Only	MDD Only	T2D Only	AD and MDD Only	AD and T2D Only	MDD and T2D Only	AD, MDD, and T2D
166	877	16	372	32	93	46

[a]Individuals with an unknown disease state for either AD, MDD, or T2D were not included in this count

Analytic Procedures

A total of 45 SNPs in *GLUL* (4 SNPs), *GLS* (8 SNPs), and *CPS1* (33 SNPs) were available in the data set for association testing. A family-based association analysis for risk of AD, T2D and MDD was performed using the PLINK DFAM procedure. Empirical p-values for single marker analyses were calculated by 100,000 permutation tests using the Max (T) permutation procedure implemented in PLINK v1.07 software (http://zzz.bwh.harvard.edu/plink/index.shtml) (Purcell et al. 2007). Haplotype analysis

was conducted in 2-SNP sliding windows using PLINK software to obtain p-values, chi-square values, and haplotype frequencies for affected and unaffected individuals. HAPOVIEW v4.2 software (https://www.broadinstitute.org/haploview/haploview) (Barrett et al. 2005) was used to determine minor allele frequencies (MAFs) and to test for Hardy-Weinberg equilibrium (HWE) using all founders in the family-based dataset. The quality control cutoff values for HWE and MAF are <0.001 and <0.05, respectively. The linkage disequilibrium (LD) structure was constructed and r^2 and D' values were determined using HAPLOVIEW. To correct for multiple testing, the Bonferroni correction ($\alpha=0.05/45=0.00111$) was used.

Bioinformatics Analysis

Because the significantly associated SNPs were all intronic, the 11 SNPs associated with AD, MDD, or T2D were input into the Human Splice Finder v3.0 program (http://www.umd.be/HSF3/) (Desmet et al. 2009) to determine if any of the SNPs may affect silencing and enhancing regions of the genes. The sequence immediately surrounding the SNP was obtained from the NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/snp). PERFECTOS-APE was used to predict transcription factor (TF) binding sites affected by the SNPs, and the hits were compared with the results of database searches for transcription factors that have been experimentally determined to be associated with the genes studied. We searched the transcription factor databases Human Transcriptional Regulation Interaction Database (http://www.lbbc.ibb.unesp.br/htri/) (Bovolenta et al. 2012) and RegNetwork

(http://www.regnetworkweb.org/) (Liu et al. 2015) for transcription factors that have been experimentally determined to interact with the genes of interest.

Results

Single Marker Analysis

PLINK single marker analysis revealed several SNPs associated with AD, MDD, and T2D with an empirical p-value < 0.05.

Alzheimer's Disease. There were four SNPs significantly associated with AD before the Bonferroni correction (rs6758866, $p = 0.00350$; rs2355570, $p = 0.03675$; rs1921907; $p = 0.00334$; and rs883844, $p = 0.00163$). All four of these SNPs are located in the *GLS* gene on chromosome 2, and all have a HWE $p > 0.001$ and a MAF of $p > 0.05$, therefore, they passed the quality control test. These results are shown in **Table 6.2**.

Table 6.2 SNPs associated with risk of AD

SNP	Position	Allele[a]	Gene	EMP1[b]	MAF[c]	HWE[d]
rs6758866	191464646	A	GLS	0.00350	0.440	0.1046
rs2355570	191489414	C	GLS	0.03675	0.258	0.3147
rs1921907	191493020	A	GLS	0.00334	0.437	0.1691
rs883844	191534014	T	GLS	0.00163	0.381	0.3054

[a]Minor allele
[b]Empirical *p*-value generated by 100,000 permutation tests using Max (T) permutation procedure in PLINK
[c]Minor allele frequency in founders
[d]Hardy-Weinberg equilibrium *p*-value

Major Depressive Disorder. Five SNPs were significantly associated with MDD before the Bonferroni correction. Four of these SNPs (rs6749597, p = 0.02776; rs9789405, p = 0.01283; rs2287602, p = 0.01692; and rs2302909, p = 0.02103) are located in the *CPS1* gene on chromosome 2, while one SNP (rs12735664, p = 0.03640) is located in the *GLUL* gene. While each of these has a MAF > 0.05, rs2302909 failed to pass the test for Hardy-Weinberg equilibrium (p = 2.0E-4). These results are shown in **Table 6.3**.

Table 6.3 SNPs associated with risk of MDD

SNP	Position	Allele[a]	Gene	EMP1[b]	MAF[c]	HWE[d]
rs12735664	180622702	C	GLUL	0.03640	0.103	0.9515
rs6749597	211128370	T	CPS1	0.02776	0.138	0.4310
rs9789405	211131628	T	CPS1	0.01283	0.146	0.7266
rs2287602	211135486	C	CPS1	0.01692	0.150	0.0344
rs2302909	211211801	A	CPS1	0.02103	0.103	2.0E-4

[a]Minor allele
[b]Empirical *p*-value generated by 100,000 permutation tests using Max (T) permutation procedure in PLINK
[c]Minor allele frequency in founders
[d]Hardy-Weinberg equilibrium *p*-value

Type 2 Diabetes. As shown in **Table 6.4**, three SNPs were significantly associated with T2D before the Bonferroni correction, two in the *GLS* gene (rs1921915, p = 0.01794; and rs1517354, p = 0.00072) and one in the *CPS1* gene (rs2302909, p = 0.00647). All of these SNPs have a MAF of > 0.05, but rs2302909 failed to pass the test for Hardy-Weinberg equilibrium (HWE p = 2.0E-4).

Table 6.4 SNPs associated with risk of T2D

SNP	Position	Allele[a]	Gene	EMP1[b]	MAF[c]	HWE[d]
rs1921915	191460572	G	GLS	0.01794	0.062	0.1093
rs1517354	191524111	C	GLS	0.00072	0.084	0.0066
rs2302909	211211801	A	CPS1	0.00647	0.103	2.0E-4

[a]Minor allele
[b]Empirical *p*-value generated by 100,000 permutation tests using Max (T) permutation procedure in PLINK
[c]Minor allele frequency in founders
[d]Hardy-Weinberg equilibrium *p*-value

Two-SNP Haplotype Analysis

PLINK two-SNP haplotype analysis revealed several haplotypes associated with AD (one haplotype), MDD (11 haplotypes), and T2D (15 haplotypes). The haplotypes with $p < 0.05$ are listed in **Table 6.5** (AD), **Table 6.6** (MDD), and **Table 6.7** (T2D).

Table 6.5 Haplotype analysis of risk of AD based on PLINK in family-based study design

Haplotype		Gene	Affected Frequency (%)	Unaffected Frequency (%)	Chi-square	*p* value
rs883844	rs10932334	GLS/CPS1				
C	T		14.34	30.55	3.894	0.0485

Table 6.6 Haplotype analysis of risk of MDD based on PLINK in family-based study design

Haplotype		Gene	Affected Frequency (%)	Unaffected Frequency (%)	Chi-square	*p* value
rs3856348	rs6725303	CPS1				
T	C		27.91	18.57	7.005	0.0081
rs6725770	rs759688	CPS1				
A	T		23.51	30.22	3.881	0.0488
rs918233	rs1509821	CPS1				
A	G		56.22	47.8	4.545	0.0330
rs17773128	rs6749597	CPS1				
C	T		14.64	8.242	5.459	0.0195
rs6749597	rs2887913	CPS1				
T	C		14.64	8.242	5.459	0.0195
rs2887913	rs9789405	CPS1				
C	T		15.52	8.242	6.748	0.0094
rs9789405	rs2287603	CPS1				
T	T		15.42	8.171	6.723	0.0095
C	T		62.9	71.56	5.157	0.0232
rs2287603	rs2287602	CPS1				
T	C		16.01	8.929	5.719	0.0168
T	T		61.72	69.64	3.925	0.0476
rs2287602	rs10515951	CPS1				
C	G		15.95	8.929	5.644	0.0175

Table 6.7 Haplotype analysis of risk of T2D based on PLINK in family-based study design

Haplotype		Gene	Affected Frequency (%)	Unaffected Frequency (%)	Chi-square	*p* value
rs1921915	rs6758866	GLS				
T	G		47.95	56.67	4.034	0.0446
rs2355570	rs1921907	GLS				
T	A		26.03	17.15	6.942	0.0084
rs1921907	rs17748089	GLS				
A	G		51.37	42.8	3.894	0.0485
rs17748089	rs1517354	GLS				
G	C		14.38	7.64	7.744	0.0054
rs1517354	rs883844	GLS				
C	C		5.37	1.02	17.06	3.63E-5
rs1509821	rs981024	CPS1				
G	C		41.78	50.64	4.105	0.0428
rs10515951	rs6714124	CPS1				
T	C		2.74	7.06	3.957	0.0467
rs2371001	rs3821135	CPS1				
A	C		2.14	6.87	4.822	0.0281
G	A		52.84	43.3	4.756	0.0292
rs3821135	rs7607205	CPS1				
C	T		6.85	13.0	4.568	0.0326
rs7607205	rs12468557	CPS1				
G	C		9.03	3.97	7.675	0.0056
rs12468557	rs2302909	CPS1				
C	A		19.01	8.87	14.89	1.14E-4
C	C		46.05	55.59	4.751	0.0293
rs2302909	rs7599931	CPS1				
A	G		5.43	2.27	5.155	0.0232
A	T		14.43	6.96	10.21	0.0014

LD Structure

The linkage disequilibrium (LD) structures for these genes are shown in **Figure 6.1**. The *GLS* and *CPS1* genes are located on chromosome 2 (2q32.2 and 2q34, respectively), while the *GLUL* gene is located on chromosome 1 (1q25.3). SNP pairs with an r^2 value > 0.5 and a D' value greater than 0.8 are listed in **Table 6.8**.

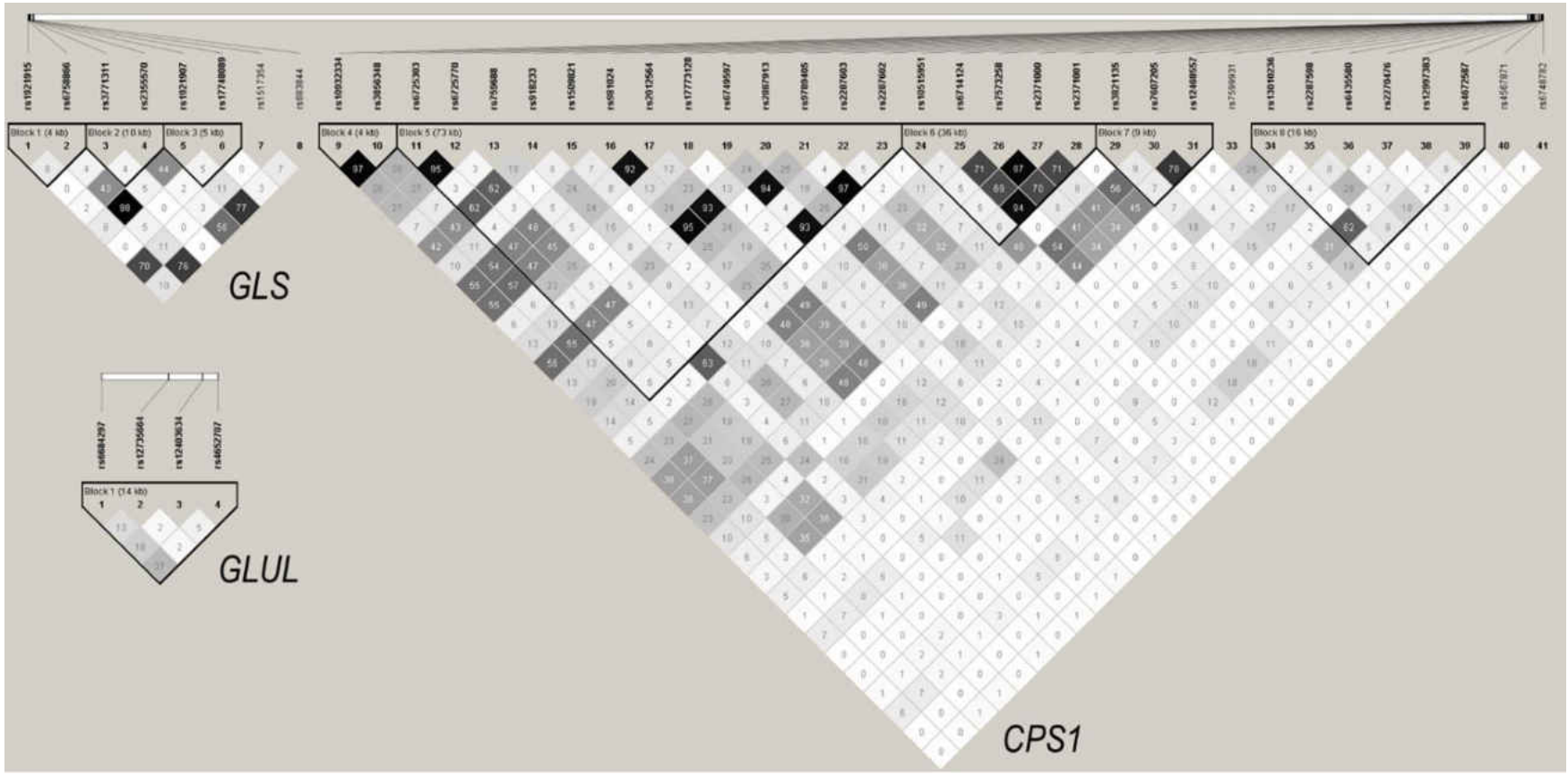

Figure 6.1: Linkage disequilibrium structure of founders from the data set using HAPLOVIEW software.

Table 6.8 Top linkage disequilibrium measures

SNP 1	SNP 2	D'	r^2
rs10932334	rs3856348	1	0.979
rs6725303	rs6725770	1	0.951
rs6749597	rs9789405	1	0.941
rs6749597	rs2287602	1	0.937
rs6714124	rs7573258	1	0.718
rs2371000	rs2371001	1	0.71
rs6725770	rs918233	1	0.622
rs6758866	rs1921907	0.997	0.984
rs2012564	rs2887913	0.997	0.932
rs981024	rs2012564	0.997	0.929
rs7573258	rs2371001	0.996	0.703
rs2355570	rs883844	0.995	0.561
rs9789405	rs2287602	0.994	0.977
rs13010236	rs12997383	0.991	0.62
rs1921907	rs883844	0.99	0.777
rs1921915	rs1517354	0.988	0.707
rs6758866	rs883844	0.987	0.764
rs7573258	rs2371000	0.986	0.97
rs6714124	rs2371000	0.982	0.695
rs6714124	rs2371001	0.981	0.947
rs981024	rs2887913	0.98	0.958
rs6725303	rs918233	0.978	0.626
rs7607205	rs12468557	0.955	0.788
rs2371001	rs7607205	0.948	0.567
rs3856348	rs2887913	0.948	0.554
rs10932334	rs2887913	0.945	0.563
rs3856348	rs981024	0.939	0.543
rs10932334	rs981024	0.936	0.551
rs6714124	rs7607205	0.935	0.542
rs3856348	rs2012564	0.931	0.571
rs759688	rs10515951	0.922	0.631
rs10932334	rs2012564	0.906	0.551
rs2887913	rs6714124	0.885	0.501

SNP pairs with D'>0.8 and r^2>0.5

Bioinformatics Analysis

All the SNPs tested are intronic. Of the 11 SNPs with a significant association with AD, MDD, or T2D, bioinformatics analysis suggests a function for seven. Of these, only 6 SNPs are in HWE; the other SNP, rs2302909, will not be considered further. Three SNPs (rs6758866, rs2355570, and rs1517354) were predicted to create enhancer sites, and one SNP decreased the likelihood of the sequence binding to transcription factors that have been experimentally determined to interact with the gene sequences of interest (rs1921907). Another two SNPs (rs9789405 and rs2287602) both created an enhancer site and decreased the likelihood of binding with a transcription factor. The details of these results are presented in **Table 6.9**.

Table 6.9 Bioinformatics analysis predicted functional effects of SNPs associated with AD, MDD, or T2D

SNP	Gene	Disease Association	Transcription Factor Binding Affected	Enhancer Site Formation
rs6758866	GLS	AD	0	+
rs2355570	GLS	AD	0	+
rs1921907	GLS	AD	ETS1	0
rs883844	GLS	AD	0	0
rs1921915	GLS	T2D	0	0
rs1517354	GLS	T2D	0	+
rs12735664	GLUL	MDD	0	0
rs6749597	CPS1	MDD	0	0
rs9789405	CPS1	MDD	E2F4	+
rs2287602	CPS1	MDD	FOXP3	+
rs2302909	CPS1	MDD/T2D	0	+

Discussion

Alzheimer's Disease

Because of the potential effect of ammonia levels on the onset and progression of AD described above (Seiler 1993), we hypothesized that SNPs in the ammonia metabolism genes *GLUL*, *CPS1*, and *GLS* may be associated with AD. However, single marker analyses only found SNP markers statistically associated with AD in the *GLS* gene (Table 6.3) in this current study. Bioinformatics analysis using the Human Splice Finder software and the PERFECTOS-APE program suggest three of these intronic SNPs may have a direct functional role in *GLS* regulation (Table 6.9). The minor alleles of SNPs rs6758866 and rs2355570 are predicted to create enhancer sites. Each of these variations may increase the expression of *GLS*. The minor allele of SNP rs1921907 is predicted by the PERFECTOS-APE software to decrease the likelihood of binding to the transcription factor ETS1. ETS1 has been shown to be expressed in brain (http://www.proteinatlas.org/ENSG00000134954-ETS1/tissue) (Uhlén et al. 2015) and to interact with the *GLS* gene (Hollenhorst et al. 2009). Because ETS1 can be either a repressor or an activator of transcription (Dittmer 2003), it is not possible to predict the direction of regulation. The statistical association of the SNP rs883844 with AD is likely the result of an indirect association. The SNP may possibly act as a marker for a nearby unsequenced variation involved in the disease process, or its association may be due to its proximity to the other three SNPs in the *GLS* gene identified in this study as being associated with AD. Data in Table 6.8 reveal rs883844 is in LD with rs2355570 ($D' = 0.995$, $r^2 = 0.561$), rs1921907 ($D' = 0.990$, $r^2 = 0.777$), and rs6758866 ($D' = 0.987$, $r^2 =$

0.764). This may explain the significant association with AD in the absence of a predicted function.

As mentioned earlier, GLS is the isoform of glutaminase mostly found in the brain and kidneys (Uhlén et al. 2015). In contrast, GLS2 is mostly localized to the liver (http://www.proteinatlas.org/ENSG00000135423-GLS2/tissue) (Uhlén et al. 2015). GLS breaks down glutamine to ammonia and glutamate. Glutamate, an excitatory neurotransmitter, is important for synaptic transmission and memory formation (Esposito et al. 2013), but increased levels can lead to excitotoxic neuronal cell death in the brain. If the observed variants in the *GLS* gene or nearby unsequenced SNPs in LD affect glutaminase levels and enzyme activity, they would affect the regeneration of the glutamate used to remove neurotoxic ammonia. Changing these glutamate levels could impact cognitive function in AD (Myhrer 1998). Several studies agree that GLS levels in AD brain are decreased (Akiyama et al. 1989; McGeer et al. 1989; Burbaeva et al. 2014). Two of the three SNPs associated with AD and predicted to have a function may create an enhancer site, but enhanced expression of *GLS* in AD is inconsistent with the published literature. It is possible that the predicted enhancer sites created are in linkage disequilibrium with nearby unsequenced SNPs that are more important for transcriptional regulation, or ETS1 usually acts as a relatively strong activator in this system. Experimental studies of autopsied AD patient brains have found an increase in glutamate levels (Xu et al. 2016). However, another study found decreased glutamate in the temporal cortex of AD brain (Gueli and Taibi 2013). Both increases and decreases in brain glutamate are associated with cognitive decline (Myhrer 1998). It is possible that changes in glutamate levels in AD

brain are region-specific and occur because of disrupted glutamate and ammonia homeostasis.

Major Depressive Disorder

Several SNPs in the *CPS1* gene were linked to MDD in this study (Table 6.3). CPS1 is critically important in ammonia metabolism as evidenced by the devastating effects of hyperammonemia in CPS1-deficient individuals (Suzuki et al. 1986; Finckh et al. 1998). Therefore, the variations identified in this study could potentially have major effects on blood ammonia levels if the variations are linked to changes in CPS1 activity. As previously mentioned, CPS1 is mainly found in the liver (http://www.proteinatlas.org/ENSG00000021826-CPS1/tissue) (Uhlén et al. 2015), and it catalyzes the incorporation of ammonia into carbamoyl phosphate in the urea cycle. The clinical observations described above suggest changes in CPS1 activity may have an impact on blood ammonia levels, and changes in blood ammonia levels may also impact glutamate levels in the brain, affecting cognition (Suárez et al. 2002). Several studies suggest changes in glutamate levels in several areas of the brain could be linked to mood disorders through disruption of the levels of glutamate and glutamine (Sanacora et al. 2012).

Bioinformatics analyses suggest two SNPs in HWE analyzed in this study may have a functional role in MDD (Table 6.9). The minor allele of the SNP rs9789405 is predicted by the Human Splice Finder program to create an enhancer site. The same SNP variant is predicted by PERFECTOS-APE to decrease the likelihood of binding by the transcription factor E2F4. E2F4 is mainly a transcriptional repressor (Crosby and Almasan 2004) and has been experimentally demonstrated to interact with the *CPS1* gene (Litovchick et al.

2007). The minor allele of the SNP rs2287602 is predicted by Human Splice Finder to create an enhancer site and by PERFECTOS-APE to decrease the likelihood of association with the transcription factor FOXP3. This transcription factor mainly plays a role in regulatory T-cell function (Vent-Schmidt et al. 2014), but it has recently been found to play a role in promoting mitochondrial oxidative metabolism (Angelin et al. 2017) and can even localize to mitochondria in hepatocytes (Rojas et al. 2016). FOXP3 is known to either increase or decrease gene expression depending upon the other transcription factors with which it associates (Szylberg et al. 2016). The Human Protein Atlas reports mRNA for both E2F4 (http://www.proteinatlas.org/ENSG00000205250-E2F4/tissue) and FOXP3 (http://www.proteinatlas.org/ENSG00000049768-FOXP3/tissue) in human liver (Uhlén et al. 2015). Based on the predicted functions of these variants, we predict there may be altered expression of *CPS1* in the liver of some MDD subjects. Altered *CPS1* expression may change the levels of ammonia in the blood, therefore changing the glutamate levels in the brains of individuals with MDD. A SNP in *CPS1* significantly associated with MDD that was not assigned a function by our bioinformatics analysis, rs6749597, is in linkage disequilibrium with the predicted functional SNPs (rs6749597:rs9789405, $D' = 1$, $r^2 = 0.941$; rs6749597:rs2287602, $D' = 1$, $r^2 = 0.937$). All variations in *CPS1* that passed the quality control tests are in the same haplotype block (Figure 6.1).

Type 2 Diabetes

SNPs in the *GLS* and *CPS1* genes were associated with T2D (Table 6.4). The SNP in the *CPS1* gene (rs2302909) is not in HWE, so it will not be further considered. The liver is the major hub of ammonia metabolism and gluconeogenesis, so it is not surprising that

ammonia metabolism genes are linked to type 2 diabetes. Excess glutamate in the liver can be deaminated, fed into the citric acid cycle, and then used for gluconeogenesis, contributing to the high blood glucose levels observed in T2D. Although T2D is traditionally associated with insulin resistance in liver and peripheral tissues, one of the hallmarks of the later stages of T2D is the inability of β-cells in the pancreas to secrete enough insulin to activate insulin signaling pathways in insulin-resistant tissues (Cantley and Ashcroft 2015). The incretin pathway is involved in insulin secretion in the pancreas (Yokoi et al. 2016). Several experimental drugs for the treatment of T2D have been designed to increase the effectiveness of the incretin pathway (Drucker et al. 2010). Glutamate has been found to increase insulin excretion by amplifying the incretin pathway in beta cells (Gheni et al. 2014). Therefore, changes in glutamate levels in the pancreas may affect the response to glucose signaling in T2D. As discussed above, CPS1, GLUL, and GLS may all affect the levels of glutamate available for signaling. GLS is expressed in pancreatic tissue (http://www.proteinatlas.org/ENSG00000115419-GLS/tissue) (Uhlén et al. 2015), so changes in expression of these genes could affect pancreatic function. These changes may play a role in the disease processes of T2D.

One SNP in HWE in the *GLS* gene is predicted to have a functional role that may be associated with T2D (Table 6.9). The SNP rs1517354 was predicted by Human Splice Finder to create an enhancer site (Table 6.9), possibly leading to an increase in *GLS* gene expression in T2D. This variation may lead to an increase in the rate at which glutamine is catabolized to glutamate and ammonia. When released from neurons, increased glutamate levels in the synaptic cleft can lead to excitotoxicity (Zhou and Danbolt 2014). A recent study suggests some of the pathology of T2D may be due to increased activity of pancreatic

receptors for glutamate (Huang et al. 2017). Glutamate excitotoxicity in the brain is mainly mediated by the NMDA receptor (NMDAR) (Lau and Tymianski 2010). A functional role for NMDARs has also been found in pancreatic beta cells (Inagaki et al. 1995; Marquard et al. 2015). The NMDAR agonist, glutamate, was found to be increased in the plasma of diabetic patients and in a rat model of diabetes (Huang et al. 2017). *In vitro* studies have shown that blocking NMDAR activation reduces glucose-mediated damage to pancreatic beta cells and improves beta cell function (Huang et al. 2017). These results suggest that changes in the expression of genes involved in glutamate metabolism may play a role in type 2 diabetes by affecting the function of pancreatic beta cells.

Changes in Expression of Ammonia Metabolism Genes: An Explanation for Some Common Pathologies in AD, MDD, and T2D?

Cortical thinning is a feature of AD (Du et al. 2007), MDD (Tu et al. 2012), and T2D (Yoon et al. 2017 Apr 27). The temporal cortex appears to be specifically affected. Cortical thinning may be caused by cell death due to glutamate excitotoxicity. As previously discussed, the epidemiology of these diseases seems to be linked. For example, a study found that individuals with AD are at increased risk for T2D (Janson et al. 2004). A more recent study concluded that comorbidity of MDD and T2D increased the risk of dementia (Katon et al. 2012).

Memantine, an NMDAR antagonist, is a drug used to reduce glutamate excitotoxicity for the treatment of AD. Memantine was also found to have protective effects on pancreatic beta cells and to reduce blood glucose levels in a mouse model of diabetes (Huang et al. 2017), and it improved some measures of cognitive functioning in a mouse model of type 2

diabetes (Iwanami et al. 2014). Double-blind studies suggest memantine may also be an effective treatment for MDD (Amidfar et al. 2017). The efficacy of this drug for the treatment of several different diseases suggests clinically significant commonalities in the disease mechanisms. Because glutamate signaling is so tightly tied to ammonia metabolism, changes in the expression of ammonia metabolism genes may be at least partially responsible for the observed cortical thinning and disease phenotypes in all three of these disorders.

Testable Hypotheses Generated and Study Limitations

This study generates several testable hypotheses: 1) Individuals with the minor allele variants of rs6758866, rs2355570, and rs1517354 have altered *GLS* gene expression and glutaminase enzyme activity. 2) Individuals with abnormal *GLS* gene expression are at greater risk for AD or T2D. 3) Individuals with the minor allele variants rs9789405 and rs2287602 have altered expression of the *CPS1* gene and altered CPS1 enzyme activity. 4) Individuals with altered *CPS1* gene expression are at higher risk for major depressive disorder. 5) Individuals with abnormal blood ammonia levels are at higher risk for major depressive disorder, and reducing blood ammonia may alleviate some of the symptoms of MDD. 6) Changes in glutamate levels in the brain due to changes in the expression of *GLS*, *GLUL*, or *CPS1* are common to AD, MDD, and T2D, and these changes contribute to the common tissue pathology observed in these diseases. 7) Drugs that regulate glutamate signaling may alleviate some symptoms of AD, MDD, and T2D.

While this study's results generate many hypotheses consistent with the published literature, the study also has several limitations. We report a genetic association from just

one data set; to decrease the risk of a type 1 error, other data sets with similar and different study designs should also be examined for comparable associations. Only rs1517354, the C-C haplotype from rs1517354 and rs883844, and the C-A haplotype from rs12468557 and rs2302909 with T2D (Tables 6.4 and 6.7) showed significant associations after a Bonferroni correction ($p < 0.00111$). Thus, our current findings might be subject to type I error, and the results need to be supported by additional large samples in a future study. Second, it was not possible to predict the direction of change in gene expression of the SNPs rs2287602 and rs1921907 because they were predicted to interact with transcription factors that can be either activators or repressors. Third, GWAS have largely not indicated ammonia metabolism gene associations with AD, T2D, or MDD. This may be because the associations are weak or because of incomplete genomic coverage in GWAS datasets. Lastly, the bioinformatics results of this study need to be supported by experimentation to verify these predictions. Even with these limitations, the findings of this study are potentially clinically relevant and warrant further investigation due to their high explanatory power and their consistency with experimental results.

Conclusions

This study used data from a family-based study design to find a novel epidemiological association of select ammonia metabolism genes with Alzheimer's disease, major depressive disorder, and type 2 diabetes. Bioinformatics analyses suggest a functional role for many of the identified SNPs. These functional roles generally fit with previously published experimental results. The associations found in this study should be confirmed by other genetic epidemiological studies to increase confidence in our

conclusions. One data set which may replicate the current results is from the Columbia University Study of Caribbean Hispanics with Familial and Sporadic Late Onset Alzheimer's Disease, dbGaP Study Accession: phs000496.v1.p1. The next step would be to experimentally verify the effects of these SNPs on gene expression and protein levels. This study is a step toward understanding the genetic and metabolic underpinnings of complex diseases with heritable components.

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CHAPTER 7

CONCLUSIONS

Hypotheses Supported

The studies reported in this dissertation investigated similarities in factors involved in the pathogenesis of several neurological diseases. Two main hypotheses are supported by this research:

(A) Amyloidogenic proteins have similarities in their residue interaction network structures that will provide insight into the formation of amyloid plaques.

(B) Altered ammonia metabolism and changes in the amount of dietary protein under certain conditions affect neurological function and play causative roles in several neurological diseases.

Specific Aim 1: Conclusions and Future Directions

To determine if amyloidogenic proteins have commonalities in their residue interaction networks and to determine how these network structures may affect amyloid-beta plaque formation when combined with 3D structural data. This aim addresses hypothesis (A).

Results from the use of lysozyme as a model for amyloidogenesis demonstrated that combining 3D structural data and RIN data can be a powerful approach in the study of amyloid formation. Novel residues were identified that are likely to be involved in its primary nucleation (residues 21, 62, 104, 122, and 112-117). Next, similar techniques were

applied to compare a group of amyloidogenic proteins to random network controls or real protein controls. Network characteristics specific to amyloidogenic proteins were identified. These characteristics may provide insight into the primary nucleation of a variety of amyloidogenic proteins. Abeta42 was analyzed in more detail, and two residues (residues 24 and 31) were identified which may be involved in preventing primary nucleation.

The results reported in this manuscript are *in silico* and are therefore preliminary. Experimental studies are needed to verify the findings. The group of amyloid proteins studied was small, so data from the analysis of more structures obtained using a variety of solvent conditions would increase confidence in the findings. Mutating the amino acid residues that we identified to be of interest for both lysozyme and Abeta42 could provide support for the conclusions drawn.

Specific Aim 2: Conclusions and Future Directions

To create a mathematical model to determine if the amount of dietary protein and degree of liver function interact to affect blood ammonia levels and to determine if the predicted changes could affect neuron health. This aim addresses hypothesis (B) and investigates the potential role of ammonia metabolism in neural function using an organismal and tissue level approach.

There is a large body of evidence that suggests Abeta contributes to oxidative damage in AD. One of the most susceptible proteins to oxidative damage in the brain is GLUL, an enzyme that functions in the removal of ammonia from the brain and other peripheral tissues. Because of the primary role of glutamate metabolism in neural function

and the toxicity of ammonia to the central nervous system, I next investigated the effects of changing the activities of enzymes involved in liver ammonia metabolism on blood and brain ammonia levels. The relationship between dietary protein intake and blood ammonia is surprisingly understudied in humans. The *in silico* model of whole-organism ammonia metabolism supports a complex interplay between the amount of dietary protein, the blood ammonia concentration, and the activities of ammonia metabolism enzymes. Protein restriction for the treatment of hepatic encephalopathy may be appropriate for some patients to limit blood and brain ammonia levels. In addition, heterozygosity for CPS1, a prevalent condition, may cause chronic, low-level hyperammonemia. Because of the high relative toxicity of ammonia on neural cells, the hyperammonemia may lead to cognitive impairment and other neurological dysfunction.

Considering the potential link between protein level in the diet and blood ammonia level, clinical studies elucidating the relationship between the two should be performed. Many of the animal studies examining the relationship used exaggerated protein diets, so studies using diets that are comparable in protein level to those consumed by humans are needed. The decreased viability of differentiated neuroblastoma cells exposed to pathological ammonium chloride levels warrants further investigation into the role of ammonia in the pathogenesis of neurological diseases.

Specific Aim 3: Conclusions and Future Directions

To determine if there is an association between single nucleotide polymorphisms (SNPs) in the GLS, CPS1, and GLUL genes and AD, MDD, and T2D, and to predict the effects of any associated SNPs in the disease processes. This aim addresses hypothesis (B) where I

suggest that altered ammonia metabolism stimulates neuropathology on a molecular and cellular level.

Data from investigating Specific Aim 2 resulted in further evidence supporting a role for pathological changes in the activities of enzymes involved in ammonia metabolism causing changes in blood ammonia levels and demonstrated that these altered ammonia levels may be toxic to neurons. Considering these results and the existing literature on the involvement of changes in glutamate and ammonia metabolism in AD, I investigated the possibility that mutations in ammonia metabolism genes were linked to AD, MDD, or T2D. SNPs in the *GLS* gene were found to be linked to AD and T2D, and SNPs in the *GLUL* and *CPS1* genes were found to be linked to MDD. Bioinformatic analysis revealed that some of these SNPs are predicted to affect the binding of certain transcription factors to the promoters of the genes, possibly affecting the levels of expression of the ammonia metabolism genes in these diseases. Since these diseases are each associated with cortical thinning, since treatment with the NMDA receptor antagonist memantine is beneficial, and since they often display comorbidity, dysfunctional ammonia metabolism may partially explain some of the common pathologies of these diseases.

To further test the hypotheses generated from the data obtained from investigating Specific Aim 3, the data from a separate set of subjects could be analyzed to test for similar associations. The predicted changes in the affinity of transcription factors for DNA sequences containing the predicted functional SNPs would lead to further insight as well. Because of the reported associations with ammonia metabolism genes, the beneficial effects of memantine and other drugs that interfere with glutamate signaling should be further evaluated for the treatment of AD, MDD, and T2D. Altered ammonia and glutamate

metabolism appears to be an important factor in the development of several neurological diseases.

Summary of Conclusions

The studies performed further elucidate several common features of neurological diseases. First, I used RINs to detect similar features of amyloidogenic proteins that have not previously been reported. Because Abeta and ammonia both cause oxidative damage in the AD brain, and GLUL, an ammonia metabolism enzyme, is especially susceptible to oxidative damage, I explored the possible role of altered ammonia metabolism and altered dietary protein intake in the regulation of systemic ammonia levels using a computational model of hepatic encephalopathy and CPS1 deficiency. As hypothesized, I found blood ammonia levels likely depend heavily on the interaction of protein diet with the activities of GLUL, GLS, and CPS1. Because high ammonia levels are toxic to neurons and ammonia metabolism genes are involved in critical glutamate signaling, I investigated the possibility that SNPs in ammonia and glutamate metabolism genes are linked to AD, MDD, and T2D. Each of these diseases had an association with GLUL, GLS, or CPS1, and some of the intronic SNPs are predicted to regulate gene expression. These associations may partially explain the common neural pathology observed in these diseases. This dissertation demonstrates that taking a broad view of neurological diseases and their causes can provide novel insights into their pathogeneses.

Personal Perspective

My undergraduate research included elucidating the interaction of Abeta with resveratrol and the interaction of insulin with phenol red. This work with amyloidogenic proteins in monomeric form sparked an interest in AD and related amyloidoses that I carried with me into my Ph.D. studies. During my graduate coursework, I realized that complex network analysis could provide a unique perspective on protein structural studies because it is a relatively new approach. I was able to find no studies that had applied complex network analysis to amyloidogenic proteins. Since there is little 3D structural and sequence similarity among amyloidogenic proteins in their soluble form, I hypothesized that there may be similarities in network connections. The results of my studies have encouraged me to continue using the approach in future research.

There is a need for more protein structural studies using network analysis. To advance the field, protein structures with well-known functions should be re-examined from a network perspective to find correlations with network metrics (Chapter 2). In addition to correlations, drug design approaches that consider residue interaction networks could be effective. For example, the results reported in Chapter 3 suggests that drugs that stabilizes the network structure of Abeta42 in a more nonpolar environment could prevent unfolding and subsequent oligomer and fibril formation. This could occur through stabilizing the predicted interaction between Val24 and Ile31. However, if stabilizing the network is the goal, perhaps other residues in the vicinity could also be a target. A network perspective could provide a wider range of drug targets not previously considered. The allosteric inhibition and activation of enzymes provides an example of this principle. The binding of an inhibitor or activator to residues away from the active site can

change both the 3D and network structures of a protein and therefore change the activity of the enzyme. Similarly, network analysis may help researchers discover previously unidentified drug targets that exert profound effects on enzyme activity even though they are not near the active site.

The series of studies on ammonia and glutamate metabolism in this dissertation was inspired by my grandfather, Wayne Birchfield. His stroke and subsequent medical care helped me understand the strong connection that exists between ammonia and glutamate metabolism and brain function. During a literature search on the cognitive effects of hyperammonemia, I found research supporting a role for ammonia in other neurological diseases, particularly AD. I came to appreciate the roles that ammonia and glutamate metabolism appear to play in a variety of neurological diseases (Chapters 4, 5, and 6).

When initiating my research, I discovered that the focus of AD research is currently on the Abeta peptide. While Abeta exerts negative effects on the brain in AD, the lack of understanding of the nature of its role in both AD and normal physiology is striking. Even the structure of Abeta and which forms in the brain are most prevalent are contested after 25 years of intense study (Wildburger et al. 2017), a testament to the complexity of AD pathogenesis.

The paucity of current treatment options should encourage researchers to examine other factors of AD pathology besides Abeta oligomerization and fibrillization (Herrup 2015). I reviewed the literature on changes in amino acid metabolism in AD (Chapter 5) and found much evidence supporting pathological changes. Furthermore, identifying these changes provides opportunities for therapeutic intervention. Researchers need to untangle which changes contribute to disease and which changes are protective adaptations to

disease processes. Treatments for AD patients may one day include dietary supplements to help alleviate some symptoms that may occur due to changes in metabolism in AD. While AD will not be cured by dietary interventions alone, they may alleviate some symptoms or delay progression of the disease.

After finishing my Ph.D. studies at East Tennessee State University, I will begin as an Assistant Professor of Biology at Mars Hill University. There I will continue research into neurological diseases and other interests. I would like to continue exploring the role of ammonia in neurological functions using *C. elegans* as a model organism. Does ammonia affect the ability of *C. elegans* to learn? (Yes, there are assays for *C. elegans* memory (Nuttley et al. 2002; Ardiel and Rankin 2010; Li et al. 2013)!) If so, what factors are involved? Because magnetic fields are effective as a treatment for some neurological diseases (Chou et al. 2015; Rutherford et al. 2015), I would also like to use *C. elegans* and *S. cerevisiae* as models for the effects of static magnetic fields on mitochondrial function (which declines in neural aging). I plan to further investigate residue interaction networks in CPS1, antimicrobial proteins, and protein toxins. I will also continue ongoing projects investigating tryptophan metabolism in AD, glutathione metabolism in AD, and pneumonia infection in AD patients. My Ph.D. research has helped me understand the power of the scientific method and an interdisciplinary approach. I will emphasize these points in my future endeavors in both research and higher education.

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